REVIEW

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GENETIC PREDICTORS OF TOXIC EFFECTS OF METHOTREXATE IN CANCER PATIENTS

Today, methotrexate (MTX) is used in combination with other medicines to treat a wide range of malignancies. Despite its proven high efficacy, MTX often causes serious side effects, which may result in the need to reduce the dose of MTX or discontinue the drug altogether. This, in turn, can provoke the development of MTX resistance and cancer progression. Predicting the risk of MTX-induced toxicity is currently difficult due to the variability of pharmacokinetics and pharmacodynamics in different patients, so the scientific literature is intensively searching for potential biomarkers. Based on the data available in the current literature, we analyzed the relationship between variants in the genes encoding the key components of MTX intracellular metabolism and the MTX-induced side effects and drug response. According to the results of our work, the most studied variants are those of the *SLC19A1* gene, which encodes the reduced folate carrier protein 1, and the *MTHFR* gene, which encodes the enzyme methylenetetrahydrofolate reductase. Studies of the effect of methylation of the promoter regions of genes on the therapeutic effect of MTX are also very promising. In conclusion, the study of molecular genetic markers of MTX toxicity is extremely relevant and necessary because it can help to avoid the effect of multidrug resistance and improve the quality of life and survival of patients.

Keywords: methotrexate, oncology, pharmacogenomics, gene, toxicity.

Methotrexate (MTX, or amethopterin) is a synthetic drug that belongs to the therapeutic group of antifolate agents and has antitumor (in high doses) and immunosuppressive (in low doses) properties. Historically, MTX is better known as a chemotherapeutic drug. After all, its successful use was first reported in the treatment of acute leukemia in children [1]. To date, MTX in combination with other medicines is used to treat hematological malignancies (acute lymphoblas-

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tic leukemia, acute myeloid leukemia, meningeal leukemia, and lymphoma), tumors of different nature (osteosarcomas, breast cancer, bladder cancer), as well as a wide range of non-cancer diseases (rheumatoid arthritis, Crohn's disease, psoriasis, multiple sclerosis, myasthenia gravis, polyarticular juvenile idiopathic arthritis, and ectopic pregnancy) [2].

By its mechanism of action, MTX is a folic acid inhibitor. Its effect on folic acid metabolism has a complex pattern that includes interaction with transporters and enzymes involved in folic acid metabolism. MTX enters the cell primarily through active transport by the human reduced folate carrier hRFC, also known as SLC19A1, or through passive membrane diffusion [3]. Once in the cell, the drug is converted to the polyglutamate MTX, which can inhibit the action of dihydrofolate reductase and several folate-dependent enzymes, such as thymidylate synthase (TYMS), 5-amino-imidazole-4-carboxamide ribonucleotide (AICAR), transformylase (ATIC), and methylenetetrahydrofolate reductase (MTHFR). This leads to a decrease in the levels of metabolically active tetrahydrofolate and its one-carbon adducts, impaired *de novo* synthesis of purines and pyrimidines, which in turn leads to the metabolic imbalance of cells, impaired proliferation, and apoptosis [2, 4].

Despite its proven high efficacy, the use of MTX, especially when administered in high doses (500 mg MTX per m^2 or more) to cancer patients, often causes serious side effects, such as bone marrow suppression, gastrointestinal toxicity, pulmonary toxicity, nephrotoxicity, hematological toxicity, and an increased risk of infections [5]. It is the manifestation of side effects that may prompt a doctor to reduce the dose of MTX or discontinue therapy altogether, which may further provoke the development of MTX resistance and cancer progression.

Predicting the risk of MTX-induced toxicity is currently difficult due to the variability of pharmacokinetics and pharmacodynamics in different patients, so the scientific literature is intensively searching for potential biomarkers. Recently, many studies have been published showing a link between the variants in genes encoding folate cycle enzymes and the MTX-induced side effects and drug response.

The aim of this review is to search, analyze, and evaluate prognostic molecular genetic markers valuable for predicting toxic effects and the lack of response to treatment when using methotrexate in cancer treatment protocols.

A literature search was conducted in the following scientific and metric databases: Elsevier, PubMed, GenBank, Google Scholar, and Springer using the keywords: methotrexate, gene variants, pharmacogenomics, and pharmacogenetics. The search depth was 5 years (2018—2023) with a retrospective review of some items down to 2013. The review includes studies published in English and Ukrainian.

Reduced folate carrier

Reduced folate carrier (SLC19A1) is an integral membrane glycoprotein belonging to the solute carrier group of transporters, which is expressed in cells of various types and provides a system of intracellular folate transport through a bidirectional anion exchange mechanism [6, 7]. SLC19A1 also actively transports antifolate chemotherapeutic agents such as MTX, raltitrexed, pemetrexed, and edatrexate into cells [7].

Among the variants of the *SLC19A1* gene encoding the reduced folate carrier, one of the most studied and widespread is the rs1051266 variant (also known as G80A, c.80A>G, or p.His27Arg, "drug response" in ClinVar). That is why research groups continue to actively study the role of this variant in the treatment of MTX.

In the study by Cwiklinska et al. [8] involving children with acute lymphoblastic leukemia treated with high doses (5 g/m^2) of MTX, it was determined that the mean steady-state concentrations of MTX were significantly higher

in 80AA homozygotes for the G80A variant of the *SLC19A1* gene. At the same time, in patients treated with MTX at a dose of 2 g/m^2, the elimination rate constant was significantly higher in 80AA homozygotes, but at a dose of 5 g/m^2, the initial elimination rate constant was significantly lower in 80AA homozygotes. The authors believe that such differences are most likely due to the saturation of MTX elimination processes. Also, a statistically significant trend towards increased transaminase levels was observed in 80AA genotype carriers, which, in the authors' opinion, is a manifestation of liver dysfunction (i.e., hepatotoxicity).

In contrast, another research group led by Esmaili et al. [9] found no significant effect of the G80A variant of the *SLC19A1* gene on the level of MTX in the blood of children with acute lymphoblastic leukemia. However, their results also indicate that the G80A variant of the *SLC19A1* gene is associated with hepatotoxicity: the risk of developing this complication was significantly increased in patients with the 80GA genotype.

In a study by Ramalingam et al. [10], in children with acute lymphoblastic leukemia receiving maintenance therapy with MTX, the presence of the 80AA genotype for the G80A variant of the *SLC19A1* gene was associated with treatment-related side effects. It should also be noted that in patients with osteosarcoma treated with high-dose MTX, the 80GG genotype was associated with better survival and a lower incidence of metastasis [11].

Other transporters of methotrexate

MTX can also be transported across the membrane intracellularly through the action of the organic anion transporter polypeptide 1B1 (fully solute carrier organic anion transporter family 1B1 member, SLCO1B1), which is predominantly expressed in the liver and intestine [12].

One of the most significant variants of the *SLCO1B1* gene, rs4149056, also known as T521C (c.521T>C, p.Val174Ala, "drug response" in ClinVar), is a nonsynonymous coding SNP, which has been shown to reduce MTX transport *in vitro* [13]. Interesting results of a genome-wide study conducted in 2013 on 1297 patients with acute lymphoblastic leukemia receiving high-dose MTX showed that the presence of the rs4149056 variant was responsible for 2% of the variability in MTX clearance. At the same time, patients with the 521CC genotype had, on average, 13% lower MTX clearance than patients with the 521TT genotype [14].

Later, similar results were obtained by other research groups. In particular, Hao et al. [15] in a retrospective study of patients with acute lymphoblastic leukemia determined that patients with the 521TT genotype at the rs4149056 variant of the *SLCO1B1* gene had higher serum concentrations of MTX after 48 and 72 h and longer delays in the elimination of this drug. In addition, patients with the 521TC or 521CC genotype had a higher risk of neutropenia.

In terms of dosing, the mean tolerated doses of MTX (defined as the average dose during the first year of maintenance therapy) were significantly lower in patients with the 521C allele at the rs4149056 variant of the *SLCO1B1* gene [16].

The presence of the rs4149056 variant of the *SLCO1B1* gene was also associated with MTX hepatotoxicity in patients with non-Hodgkin's lymphoma (who mostly received MTX at a dose of 5 g/m^2): patients with the 521TC and 521CC genotypes had a higher incidence of hepatotoxicity than patients with the 521TT genotype [17]. Indirectly, the same results were obtained in the study by Yang et al. [18] in pediatric acute lymphoblastic leukemia: the number of rescue doses of leucovorin in patients with genotypes 521CC and 521CT was higher than in patients with 521TT genotype.

Regarding long-term prognosis, an assessment of 5-year relapse-free survival in children with acute lymphoblastic leukemia showed that the long-term outcome was worse in patients

with the 521CC genotype at rs4149056 variant of the *SLCO1B1* gene [19].

In contrast, in a study conducted by Razali et al. [20] involving children with acute lymphoblastic leukemia, no significant effect of the rs4149056 variant of the *SLCO1B1* gene on the MTX clearance and side effects after the use of this drug was found. Most likely, such discrepancies in the results can be explained by the small size of the group studied by Razali et al.

It should be noted that MTX is also a substrate for transporters of another type, namely ATP-binding cassette transporters (ABC transporters), which mediate the unidirectional active membrane movement of a wide range of molecules using the energy generated during the ATP hydrolysis [21]. ABCB1, member 1 of ATP-binding cassette subfamily B, which belongs to the ABC transporter superfamily, is one of the main efflux transporters of drugs known to cause multidrug resistance in cancer patients receiving chemotherapy [22].

Among the variants of the *ABCB1* gene of the same name, the most common and well-studied variant is rs1045642 (also known as C3435T, c.3435T>C, "benign" in ClinVar), which is a synonymous variant formed by the substitution of cysteine for thymine at position 3435. It should be noted that this variant is common among the population of Ukraine [23].

In a study by Han et al. [24] involving patients with various types of malignant hematological diseases, the association of 3435CC genotype at the rs1045642 variant of the *ABCB1* gene with a higher risk of hematological toxicity at an MTX dose of 2.5—5 g/m^2 was determined. In contrast, another study group did not observe a significant association between the rs1045642 variant of the *ABCB1* gene and various types of toxicity when taking high doses of MTX in patients with hematological malignancies [25]. However, they found that carriers of the 3435T allele of the *ABCB1* gene had a 10-fold higher risk of delayed MTX elimination. Similar results were obtained by Zhou et al. [26] in patients with the 3435TT genotype, who showed delayed clearance of MTX and higher serum creatinine levels. In pediatric patients with acute lymphoblastic leukemia, lymphoma, or osteosarcoma, in the presence of the 3435T allele of the *ABCB1* gene, an almost 2.5-fold increase in the risk of mucositis was noted with MTX administration [27].

Dihydrofolate reductase

DHFR catalyzes the reduction of dihydrofolate to tetrahydrofolate. Tetrahydrofolate is further used as a cofactor for folate-dependent enzymes and is therefore essential for many biosynthetic pathways including amino acid and nucleic acid formation, and methylation [28]. Due to the important biological role of DHFR, changes in its expression level can affect susceptibility to various diseases and sensitivity to anticancer drugs. Changes in *DHFR* gene expression can occur in the presence of certain genetic variants. As mentioned above, this enzyme is the main target of MTX, and therefore its features play an important role in the development of drug resistance and can lead to a decrease in treatment effectiveness or side effects [28].

However, only a few studies have examined the relationship between *DHFR* gene variants and side effects resulting from high-dose MTX. To date, the largest study is the one conducted by Ceppi et al. [29] on 404 children with acute lymphoblastic leukemia, where they analyzed the impact of 6 *DHFR* gene variants on the treatment outcomes. The researchers demonstrated that only the rs442767 (also known as C680A, c.237+713G>A, not reported in ClinVar), rs408626 (also known as A317G, c.237+350T>C, not reported in ClinVar), and rs1650697 (also known as C35T or G473A, c.-473T>C, "bening" in ClinVar) variants had a significant effect. In particular, the presence of the 680CC genotypes for the rs442767 variant, 317AA for the rs408626 variant, and 35TT for the rs1650697 variant re-

sulted in the shortest event-free survival time of the patients in the high-risk group. In another study, the authors found that the presence of the 1610CC genotype at the rs1650694 (also known as C-1610G/T, c.358+76G>A, "benign" in ClinVar) variant of the *DHFR* gene was associated with a higher risk of hematological toxicity, and the 680CC genotype at the rs442767 variant was associated with a 5-fold increased risk of hepatotoxicity [30]. The opposite results were obtained by a group of scientists led by Guillermo Gervasini [31], who showed that carriers of the 680AA genotype with the rs442767 variant had a higher white blood cell count in the therapeutic range, more episodes of neutropenia, a higher number of blood tests with elevated levels (> 400 mg/dL) of lactate dehydrogenase, and more treatment interruptions due to the toxicity. Perhaps this difference can be explained by the fact that the study [11] involved children with acute lymphoblastic leukemia who were on MTX maintenance therapy.

Also, there are interesting results of the study by Tulstrup et al. [32] who analyzed the results of whole-genome sequencing in 447 patients with acute lymphoblastic leukemia and the level of MTX metabolites. It should be noted that the level of MTX metabolites, according to the authors, is the main pharmacological marker of methotrexate anti-leukemia efficacy and cytotoxicity. As a result of this work, 6 significant variants in the *DHFR* gene were identified, with rs1382539 (c.238-76G>A, "benign" in ClinVar) having the greatest effect on metabolite levels and being in strong linkage disequilibrium with five other significant variants: rs1478834, rs863215, rs1650666, rs1677703, and rs1222809 [32].

Thymidylate synthase

Thymidylate synthase is a central enzyme in purine synthesis and catalyzes the conversion of deoxyuridine monophosphate (dUMP) and 5,10-methylenetetrahydrofolate (5,10-MTHF) to deoxythymidine monophosphate (dTMP) and dihydrofolate (DHF). Subsequently, dTMP is phosphorylated to deoxythymidine triphosphate (dTTP) and used for DNA synthesis and repair [33]. Thymidylate synthase also functions as an RNA-binding protein, which means it can function as a translational regulator of gene expression [34].

In a study involving 133 children with acute lymphoblastic leukemia, an increased risk of vomiting and hepatotoxicity was found in the presence of the 3R/3R genotype at the 2R3R (rs34743033, not reported in ClinVar) variant of the *TYMS* gene [8]. Another study identified an increased risk of hepatotoxicity in the presence of the DD genotype at rs16430 (or 3'UTR 6-bp ins/del, note: this variant is under review at dbSNP) of the *TYMS* gene [35]. At the same time, in another study, the aforementioned *TYMS* gene variants were not associated with oral mucositis caused by high doses of MTX [36].

ATIC

5-amino-imidazole-4-carboxamide ribonucleotide transformylase is an enzyme that catalyzes the addition of a formyl group to AICAR to form formyl AICAR (FAICAR) in the last stages of *de novo* purine synthesis [37]. The inhibition of ATIC by MTX leads to intracellular accumulation of AICAR and its metabolites and an increase in the adenosine levels. Adenosine may be responsible for the antiproliferative effect of MTX in cancer patients [38].

Park et al. [39] demonstrated the association of the 347C>G (rs2372536, c.347C>G or p.Thr116Ser, "benign" in ClinVar) variant of the *ATIC* gene with a good histological response to chemotherapy in patients with osteosarcoma. As for the effect of this gene on the risk of toxic effects, a recent study by Gong et al. [37] found no significant effect of *ATIC* gene variants (rs2372536, rs4673993, rs12995526, and rs7563206) on the risk of mucositis in children

with acute lymphoblastic leukemia, lymphoma, or osteosarcoma.

Methylenetetrahydrofolate reductase

Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme in the folate cycle. It catalyzes the irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is a methyl group donor for the conversion of homocysteine to methionine [40]. Since MTHFR plays a crucial role in folic acid metabolism, there are many studies investigating the effect of the *MTHFR* gene variants on the efficacy and side effects of MTX in cancer patients.

Among the variants of the *MTHFR* gene, the most common and significant are C677T (rs1801133, c.665C>T or p.Ala222Val, "drug response" in ClinVar) and A1298C (rs1801131, c.1286A>C, or p.Glu429Ala, "conflicting interpretations of pathogenicity" in ClinVar), which are associated with a decrease in MTHFR activity. The presence of the C677T variant of the *MTHFR* gene was associated with delayed MTX clearance and lower platelet counts after high-dose MTX treatment in children with acute lymphoblastic leukemia [26]. Moreover, the authors noted that after treatment, children with the 677TT genotype had significantly elevated serum levels of L-phenylalanine, which can cause toxic side effects. Another research group, basing on the results of their studies, found that the presence of the 677T allele at variant C677T or the 1298AA genotype at variant A1298C is associated with a higher risk of hematological toxicity in adults with malignant hematological diseases during high-dose MTX therapy [24]. Another group of authors also identified a significant impact of *MTHFR* gene variants: the 677TT genotype at the C677T variant was associated with higher plasma MTX levels after 48 h, a risk of hematological toxicity, and the need for more medical interventions (MTX dose reduction, MTX discontinuation, or hospitalization for hematological and hepatic toxicity); and the 1298AC genotype at the A1298C variant was associated with a reduced risk of hepatotoxicity [9]. In contrast, in the study by Karschnia et al. [41], the presence of the 1298AC genotype at variant A1298C was associated with an increased incidence of treatment-induced leukoencephalopathy and decreased overall survival in patients with primary central nervous system lymphoma treated with MTX-based regimens.

The results of a large prospective study of 271 children with acute lymphoblastic leukemia showed that children who carried the 677T allele (genotype 677CT or 677TT) of the C677T variant had a higher risk of hepatotoxicity and mucositis, but at the same time, a reduced risk of neutropenia [42]. Similar results were obtained by a group of scientists led by Yang et al. [43]. Children with acute lymphoblastic leukemia who carried the 677CT or 677TT genotypes had higher risks of leukemia, neutropenia, anemia, and hepatotoxicity when receiving high-dose MTX.

Epigenetic processes

The epigenetic processes, in particular, the state of methylation of the promotor region of genes, represent an important aspect in the implementation of the therapeutic effect of MTX. DNA methylation is a covalent chemical modification resulting from the addition of a carbon methyl group at position 5 to the cytosine ring. DNA methylation at CpG sites of the promoter islands can affect gene expression.

Some authors have investigated the methylation status of the *SLC19A1* gene in pediatric patients with acute lymphoblastic leukemia treated with high-dose MTX, which allowed them to draw conclusions about the response to the therapy [44]. In particular, the level of hypermethylation of the CpG_10 unit in the promoter region of the *SLC19A1* gene showed a significant positive correlation with the plasma concentration

of MTX 24 h after the start of therapy. Similar results were obtained by another research group in adult patients with acute lymphoblastic leukemia with slowed MTX elimination: higher methylation levels were observed in the promoter region of the *SLC19A1* gene [45].

Patients with primary breast cancer treated with cytotoxic chemotherapy (most often with cyclophosphamide, MTX, and fluorouracil) and having a high level of methylation of the *BRCA1* gene promoter region had significantly better survival [46]. It should be noted, given the high frequency of hypermethylation of the promoter regions of the *BRCA1* and *BRCA2* genes in women with breast cancer [47, 48] compared to pathogenic variants of these genes, further research in this area is quite promising. Moreover, the study of gene methylation patterns will contribute to better prediction of toxic effects and response to treatment with MTX.

Conclusions

MTX is an effective drug for cancer treatment but the occurrence of side effects limits its use. Mucositis, hepatotoxicity, gastrointestinal side effects, and myelosuppression are the main side effects of high-dose MTX polychemotherapy and often require a dose reduction or complete drug withdrawal.

Progress in the field of genetics and the emergence of the concept of pharmacogenomics will allow the use of personalized treatment regimens in medical practice, which will help doctors predict the response to therapy and avoid side effects. As for the pharmacogenetics of MTX in cancer patients, the current scientific literature contains somewhat contradictory data. However, the study of molecular genetic markers of MTX toxicity is extremely relevant and necessary to avoid the effect of multidrug resistance and improve the quality of life and survival of patients.

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ГЕНЕТИЧНІ ПРЕДИКТОРИ ТОКСИЧНИХ ЕФЕКТІВ ПРИ ЗАСТОСУВАННІ МЕТОТРЕКСАТУ В ПАЦІЄНТІВ З ОНКОЛОГІЧНОЮ ПАТОЛОГІЄЮ

На сьогоднішній день метотрексат (МТХ) у комбінації з іншими лікарськими засобами застосовується для лікування широкого кола злоякісних новоутворень. Незважаючи на його доказану високу ефективність, застосування МТХ часто викликає серйозні побічні явища, що може призвести до необхідності зниження його дози або повної відміни. Це, у свою чергу, може спровокувати розвиток резистентності до МТХ та прогресування онкологічного захворювання. Прогнозування ризику виникнення МТХ-індукованої токсичності в даний момент ускладнене через варіабельність фармакокінетики та фармакодинаміки в різних пацієнтів, тому в науковій літературі спостерігається інтенсивний пошук потенційних біомаркерів. На основі сучасних літературних даних нами проаналізовано зв'язок між варіантами генів, що кодують ключові ланки внутрішньоклітинного метаболізму МТХ, та побічними ефектами, спричиненими МТХ, а також відповіддю на прийом препарату. Як показали результати нашої роботи, найбільш досліджуваними є варіанти гена *SLC19A1*, що кодує переносник відновлених фолатів, та гена *MTHFR*, що кодує фермент метилентетрагідрофолатредуктазу. Також дуже перспективними є дослідження впливу метилювання промоторних ділянок генів на реалізацію терапевтичної дії МТХ. На завершення, дослідження молекулярно-генетичних маркерів токсичності МТХ є надзвичайно актуальним і необхідним, адже це дозволить уникнути ефекту множинної резистентності та покращити якість життя і виживаність пацієнтів.

Ключові слова: метотрексат, онкологія, фармакогеноміка, ген, токсичність.