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CHANGES IN *DNMT1* EXPRESSION AS A MARKER OF EPIGENETIC REGULATION DISTURBANSES IN MULTIPLE SCLEROSIS PATIENTS

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BACKGROUND: Multiple sclerosis is a chronic neurodegenerative autoimmune disease characterized by the presence of foci of inflammation and demyelination in the central nervous system. The initiation of pathological processes in multiple sclerosis is caused by a complex interaction of genetic factors, unfavorable environmental factors and epigenetic influences. Progressive neurological symptoms caused by axonal conduction disorders, axonal death and neurodestruction lead to a significant decreased patients' quality of life and disability. The search for a new markers to improve diagnostic and therapeutic methods, including taking into account the genetic background and epigenetic interactions, is an urgent task.

AIM: The work was aimed to study the changes in *DNMT1* mRNA expression in multiple sclerosis patients with different disease duration, to analyze methylation of *DNMT1* promoter, and compare the changes in the level of *DNMT1* expression with the homocysteine content in the blood, and the presence of polymorphic variants in genes coding the key folate cycle enzymes.

MATERIALS AND METHODS: The level of *DNMT1* mRNA expression in peripheral mononuclear blood cells was assessed by reversed transcription followed by polymerase chain reaction. Fluorescent polymerase chain reaction followed by methyl-sensitive analysis of high-resolution melting curves was used to analyze methylation of the *DNMT1* promoter. The content of homocysteine in the blood was determined by chemiluminescence immunoassay. The real-time polymerase chain reaction was used for genotyping by polymorphism of folate cycle genes; the fluorescent probes with the LNA modifications were used to discriminate alleles.

RESULTS: It has been shown that in multiple sclerosis patients, including those at the onset of the disease, the level of *DNMT1* mRNA expression is significantly lower than in the control group. No relationship was found between the decrease in *DNMT1* expression and the level of promoter methylation. Strong positive relationship between the level of *DNMT1* mRNA expression and homocysteine content in patients with multiple sclerosis and the combined effects of the genotypes of *MTR* A2756G and *MTHFR* C677T polymorphism on the expression of *DNMT1* have been shown. These findings suggest that genetically determined features of folate metabolism may contribute to the disruption of epigenetic regulation in multiple sclerosis.

CONCLUSIONS: The obtained results indicate the promise of research aimed to identifying the factors causing epigenetic changes in multiple sclerosis. Studying the mechanisms of the folate cycle genes polymorphic variants contribution to the pathogenesis of multiple sclerosis could be one of the possible ways to improve diagnostic and therapeutic approaches.

Keywords: multiple sclerosis; epigenetics; methylation; *DNMT1*; gene polymorphism; folate cycle.

ИЗМЕНЕНИЕ ЭКСПРЕССИИ *DNMT1* КАК МАРКЕР НАРУШЕНИЯ ЭПИГЕНЕТИЧЕСКОЙ РЕГУЛЯЦИИ У ПАЦИЕНТОВ С РАССЕЯННЫМ СКЛЕРОЗОМ

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Обоснование. Рассеянный склероз — хроническое нейродегенеративное аутоиммунное заболевание, характеризующееся наличием очагов воспаления и демиелинизации в центральной нервной системе. Запуск патологических процессов при рассеянном склерозе обусловлен сложным взаимодействием генетических факторов, неблагоприятных факторов среды и эпигенетическими влияниями. Прогрессирующая неврологическая симптоматика

Abbreviations

DNMTs, DNA methyltransferases; MS, multiple sclerosis; MTHFR, methylenetetetrahydrofolate reductase; MTR, methionine synthase; MTRR, methionine synthase reductase; PCR, polymerase chain reaction; MS-HRM, methyl-sensitive high-resolution melting curve analysis; EDSS, expanded disability status scale.

вследствие нарушений аксональной проводимости, гибели аксонов и нейродеструкции приводит к значительному ухудшению качества жизни пациентов и инвалидизации. Поиск новых маркеров для совершенствования методов диагностики и терапии, в том числе с учетом генетического профиля и эпигенетических взаимодействий, является актуальной задачей.

Цель — исследование изменений экспрессии мРНК *DNMT1* у пациентов с рассеянным склерозом с разной продолжительностью заболевания, анализ метилирования промоторной области гена *DNMT1* и сопоставление изменений в уровне экспрессии *DNMT1* с содержанием гомоцистеина в крови и наличием полиморфизмов генов, кодирующих синтез ключевых ферментов фолатного цикла.

Материалы и методы. Уровень экспрессии мРНК *DNMT1* в периферических мононуклеарных клетках крови оценивали методом обратной транскрипции с последующей полимеразной цепной реакцией, для анализа метилирования промотора *DNMT1* использовали метод флуоресцентной полимеразной цепной реакции с метил-чувствительным анализом кривых плавления с высоким разрешением. Содержание гомоцистеина в крови определяли методом иммунохемилюминесцентного анализа. Для генотипирования по полиморфизмам генов фолатного цикла использовали метод полимеразной цепной реакции в реальном времени, для дискриминации аллелей применяли флуоресцентные зонды с LNA-модификациями.

Результаты. Показано, что у пациентов с рассеянным склерозом, в том числе в дебюте заболевания, уровень экспрессии мРНК *DNMT1* достоверно ниже, чем у добровольцев контрольной группы. Связи между снижением экспрессии *DNMT1* и уровнем метилирования промотора обнаружено не было. Выявленная сильная положительная взаимосвязь между уровнем экспрессии мРНК *DNMT1* и содержанием гомоцистеина у пациентов с рассеянным склерозом и наличие сочетанного влияния генотипов по полиморфизмам A2756G гена *MTR* и C677T гена *MTHFR* на экспрессию *DNMT1* позволяет предполагать, что генетически обусловленные особенности метаболизма фолатов могут способствовать нарушению эпигенетической регуляции при рассеянном склерозе.

Заключение. Полученные результаты указывают на перспективность исследований, направленных на выявление факторов, обусловливающих эпигенетические изменения при рассеянном склерозе. Изучение механизмов, определяющих вклад полиморфных вариантов генов фолатного цикла в патогенез рассеянного склероза, — один из возможных путей совершенствования диагностических и терапевтических подходов.

Ключевые слова: рассеянный склероз; эпигенетика; метилирование; *DNMT1*; полиморфизм генов; фолатный цикл.

Background

Disruption of epigenetic regulation represents a crucial link in the pathogenesis of multifactorial diseases by mediating the interaction of environmental factors and genetic predisposition [1, 2]. DNA methylation, the transfer of a methyl group from the universal methyl donor S-adenosine methionine to cytosine, which is part of CpG dinucleotides, was the first to be discovered and is the most studied mechanism of epigenetic control [3]. This reaction is catalyzed by enzymes of the DNA methyltransferase family (DNMTs) [4, 5]. The *DNMT1* gene product provides stability of methylation patterns during cell division as it has affinity primarily for half-methylated DNA. Methyltransferases DNMT3a and DNMT3b perform *de novo* methylation and are expressed primarily in undifferentiated embryonic cells [6].

The activity of methyltransferases is altered in several pathological processes, including tumor growth, neurodegenerative diseases, and autoimmune pathology. For several malignant neoplasms, there is a correlation between the suppression of oncosuppressor gene production and hyperactivation of DNMTs at both the transcriptional and translational levels [7–9]. Furthermore, alterations in the activity of DNA methyltransferases, in conjunction with alterations in the genome-wide methylation levels and activation or inactivation of specific genes involved in the pathogenesis of numerous diseases of the central nervous system, have been demonstrated. Particularly, low *DNMT1* expression in patients with Alzheimer's disease correlates with high production of presynilin, whereas dysregulation of α-synuclein expression, *DNMT1* translocation, and hyperproduction of genes involved in its pathogenesis have been associated with Parkinson's disease [10–12]. There is a correlation between the formation of pathological protein aggregates, reduced *FUS* gene expression, and *DNMT1* overexpression in patients with one of the variants of familial amyotrophic lateral sclerosis [13].

Multiple sclerosis (MS) is a progressive demyelinating autoimmune disease characterized by a chronic course with increasing neurological deficits [14]. Significant changes in DNA methylation profiles occur in MS, including hypomethylation of promoters of genes associated with the control of myelination, T-lymphocyte differentiation, and inflammatory reactions (PAD2, FOXP3, and IL-17A) [15–18]. As a notable decline in *DNMT1* mRNA expression is observed in MS patients [19], it can be hypothesized that the suppression of *DNMT1* activity contributes to the formation of aberrant methylation patterns and dysregulation of gene expression. Thus, it is of interest to compare *DNMT1* gene expression levels in patients at the onset of MS and during the long-term course of the disease. Moreover, it is necessary to ascertain whether the suppression of *DNMT1* expression is associated with alterations in the degree of methylation of the promoter region of the gene, or whether this reduction is because of other mecha-

nisms. Particularly, the regulation of methylation processes is closely associated with the functioning of the folate cycle and activity of enzymes controlling the metabolism of homocysteine and methionine and formation of methyl donors [20, 21]. Excessive accumulation of homocysteine because of its slow conversion into methionine is accompanied by a decrease in the production of S-adenosine methionine, a deficiency of methyl groups, and a decrease in the ratio of S-adenosine methionine/S-adenosine homocysteine [22]. S-adenosine homocysteine, a byproduct of the methylation reaction, functions as a competitive inhibitor of DNMTs [23]. Consequently, the peculiarities of folate metabolism and homocysteine metabolism may be affected by both insufficient intake of micronutrients, especially B vitamins, which are coenzymes of the folic acid cycle, and genetically determined decreases in the activity of key genes of the folate cycle, including methylenetetrahydrofolate reductase (*MTHFR*), methionine synthase (*MTR*), and methionine synthase reductase (*MTRR*).

The aim of this study was to assess the level of *DNMT1* mRNA expression and degree of *DNMT1* promoter methylation in MS patients with various disease durations, and to determine the relationship between *DNMT1* gene activity and the presence of folate cycle gene polymorphisms.

Materials and methods

An experimental group of 98 patients with MS, diagnosed according to the McDonald criteria [24], and a control group of 32 healthy volunteers without neurologic pathology were obtained for the study (Table 1). The patients included in the study were under outpatient observation at the clinic of the Institute of Experimental Medicine and the Almazov National Medical Research Center. Venous blood samples for molecular biological studies were obtained from patients and healthy volunteers after they signed a voluntary informed consent form.

Genetic testing was conducted to detect polymorphisms of folate cycle genes, including *MTHFR, MTR*, and *MTRR*, in all participants. A group of 30 individuals were selected from the total sample to assess *DNMT1* expression.

Genotyping for polymorphisms C677T and A1298C of the *MTHFR* gene, A2756G of the *MTR* gene, and A66G of the *MTRR* gene

Venous blood samples were collected in vacuum tubes containing an anticoagulant (ethylenediaminetetracetic acid) for subsequent molecular biological and genetic analyses. Nuclear DNA was isolated from whole blood according to standard protocols using the DNA Sorb B reagent kit (Next-Bio LLC, St. Petersburg) for genotyping. Genotypes were determined by polymerase chain reaction (PCR) using specific oligonucleotide primers and allele-specific LNA-modified fluorescent probes for the following polymorphisms: C677T and A1298C of the *MTHFR* gene (SNP rs1801133 and rs1801131), A2756G of the *MTR* gene (SNP rs1805087) and A66G of the *MTRR* gene (SNP rs1801394). The primers and probes were synthesized from DNA Synthesis LLC.

Analysis of expression level and evaluation of methylation of *DNMT1* gene promoter

Three groups consisting of ten patients diagnosed with MS (disease duration of >1year), ten patients in the MS onset (disease duration of no more than 6 months), and ten healthy volunteers of an appropriate age, were selected for determining *DNMT1* expression.

The mRNA expression levels and degree of methylation of the promoter region of the *DNMT1* gene were assessed using peripheral blood mononuclear cells isolated by gradient centrifugation with the Proba-Ficoll reagent kit (DNA-Technology LLC, Russia) according to the manufacturer's instructions.

Extraction of mRNA and evaluation of *DNMT1* gene expression level

Total mRNA was isolated from peripheral blood mononuclear cells using the Extract-RNA reagent (Evrogen, Russia). The mRNA expression level of

Table 1 / Таблица 1

Demographic characteristics of control subjects and multiple sclerosis patients. Data are presented as: median (1rd quartile; 3rd quartile)

Демографические характеристики контрольной группы и пациентов с рассеянным склерозом. Данные представлены в виде: медиана [1-й квартиль; 3-й квартиль]

Indicators	Control group, *n* = 32	Patients with multiple sclerosis, *n* = 99
Gender (W : M)	24 : 8	71 : 28
Age (years)	37,0 [31,5; 47,0]	40,0 [32,0; 48,0]
Age of MS onset (years)	–	31,0 [24,0; 37,0]
EDSS score	–	3,8 [2,0; 5,1]

N o t e: EDSS, expanded disability status scale.

the *DNMT1* gene was assessed by reverse transcription followed by PCR using specific oligonucleotide primers and TaqMan fluorescent probes, with real-time recording of the results. The expression level of *DNMT1* was calculated using the ΔΔCt method relative to the expression level of the β-glucuronidase beta (GUSB) gene. The stability of its expression level and validity of its use as an internal control in the study of gene expression in human peripheral blood mononuclear cells were demonstrated previously [25].

Extraction of genomic DNA and determination of the degree of methylation of the promoter of the *DNMT1* gene

Genomic DNA was isolated from peripheral mononuclear cell samples using the sorbent extraction method (DNA-Technology Ltd.), following the manufacturer's instructions. The concentration of the extracted genomic DNA was determined using spectrophotometer at a wavelength of 260 nm on a NanoDrop LITE instrument (ThermoFisher Scientific, USA), according to the manufacturer's instructions. The absorbance ratio at 260 and 280 nm wavelengths (A260/280) was used to assess the purity of the preparation.

At least 100 ng of DNA from each sample was subjected to bisulfite conversion using the BisQuick reagent kit (Evrogen, Moscow) to convert unmethylated cytosines to uracil. All samples (including samples of methylated and unmethylated control DNA, whose algorithm is described below) were processed simultaneously to avoid potential batch effects.

The efficiency of bisulfite conversion was assessed using an artificial sample of completely unmethylated human DNA in the form of a PCR-amplified product including a 638-bp region in the promoter region of the *DNMT1* gene. The primers used for amplification were as follows: forward (638_F): 5′-GGGAATCCACGGTCCATTT-3′ and reverse (638_R): 5′-GGGCTTCTTCTCGCTGCTGCTGCTTTAT3′.

Assessment of methylation of the promoter region of the *DNMT1* gene

Fluorescent PCR followed by methyl-sensitive high-resolution melting curve analysis (MS-HRM) was used to determine the extent of methylation of the *DNMT1* promoter. The high-resolution melting curve analysis technique, initially developed for genotyping, was adapted to assess site-specific methylation. The data obtained by quantitative assessment of methylation using MS-HRM analysis are comparable to those obtained by pyrosequencing, which validates the method [26].

The website https://www.ebi.ac.uk/Tools/seqstats/emboss_cpgplot/ was used to identify sites within the promoter region of the *DNMT1* gene exhibiting a high content of CpG dinucleotides and to select specific primers. When estimating the degree of methylation using HRM analysis, we selected primers that allowed us to avoid errors associated with preferential amplification of unmethylated and/or incompletely converted DNA [27, 28]. Additionally, the alterations that occur during bisulfite conversion were detected according to the criteria delineated in the manual accessible at https://zymoresearch.eu/pages/bisulfite-beginner-guide. Two variants of methylation-independent primers proposed by F. Coppedè et al. [29] for analyzing the methylation level of the promoter region of the *DNMT1* gene were used. The primer sequences are presented in Table 2 and the position of the primers on the sequence is schematically depicted in Fig. 1.

The *DNMT1* promoter region was amplified using the Roche LightCycler 96 platform (Roche AppliedScience, Laval, PQ, Canada). A previously described protocol [30] was used for PCR and subsequent melting curve analysis. Protocol parameters for all staging conditions were as follows: 1 cycle of 95°C for 12 min, 60 cycles of 95°C for 30 s, 63°C for 30 s, and 72°C for 45 s; followed by HRM 95°C for 10 s and 50°C for 1 min, 65°C for 15 s, and continuous measurement to 95°C with one measurement at 0.2°C. PCR was conducted in a final volume of 50 μl using hot-start TaqM polymerase (AlkorBio LLC), 20 pmol of each primer, and 10 ng of bisulfite-modified DNA matrix. All reactions were performed in triplicate.

Samples with various percentages of methylation were prepared from 100% methylated and unmethylated human DNA control in appropriate ratios and

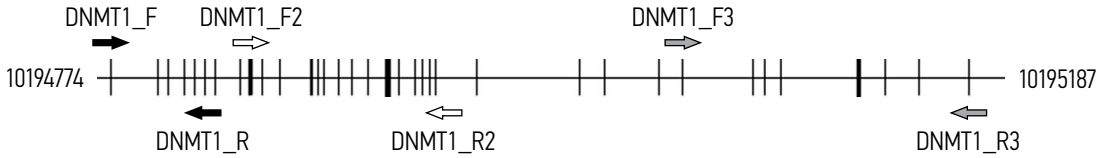

Fig. 1. Schematic representation of the *DNMT1* promoter region and the positions of the primers used to amplify the promoter region. Cross lines indicate the position of CpG dinucleotides (double lines correspond to CGCG regions). The numbers indicate positions on the chromosome for the sequence NC_000019.10 located in the NCBI

Рис. 1. Схематическое изображение промоторной области *DNMT1* и положение праймеров, использованных для амплификации участков промотора. Поперечные линии показывают положение CpG-динуклеотидов (двойные линии соответствуют участкам CGCG). Цифрами обозначены позиции на хромосоме для размещенной в банке данных NCBI последовательности NC_000019.10

Table 2 / Таблица 2

The sequences of oligonucleotide primers for bisulfite-converted DNA used to analyze the level of methylation of the *DNMT1* promoter region using the MS-HRM method

Последовательности олигонуклеотидных праймеров для конвертированной бисульфитом ДНК, использованные для анализа уровня метилирования промоторной области гена *DNMT1* методом MS-HRM

Sequence 5′–3′	Fragment length	CpG sites (*n*)	Region relative to TSS	Positions on a chromosome
DNMT1_F GCGTTTTGTTTGTTTTTT	106	9	47–151	10194773–10194878 NC_000019.10
DNMT1_R CCCAAATACCCACACTAA				
DNMT1_F2 ACGGTTAGTGTGGGTATT	155	21	–87–67	10194857–10195012 NC_000019.10
DNMT1_R2 CCAAACTAAAATAATAAA				
DNMT1_F3 GGTATCGTGTTTATTTTTTAGTAA	114	9	–263÷–149	10195084–10195187 NC_000019.10
DNMT1_R3 ACGAAACCAACCATACCCAA				

used for calibration. Fully methylated DNA was obtained by reaction with the enzyme CpG-methylase M.SSI (SibEnzyme LLC) from genomic DNA of the human cell line Raji (Evrogen JSC). The amplified PCR product corresponding to the investigated *DNMT1* promoter region was used as a sample of fully unmethylated DNA.

The control and test DNA samples were simultaneously subjected to bisulfite conversion. The effective DNA concentration was normalized by pre-PCR to ensure that the difference in Ct threshold cycles for methylated and unmethylated DNA did not exceed two cycles. The procedure involved preparing standard samples (calibrators) with a specified percentage of methylation, ranging from 0% to 100%. Calibrators were included in each run, and the values obtained from them were used to construct standard curves and determine the methylation level for each of the samples under study.

Post-processing of MS-HRM data

The selection of the postprocessing method for MS-HRM data was informed by approaches used in previous studies. The chosen calculation method involves calculation of the Area Under Curve (AUC), which is a derivative of the HRM curve, and comparing AUC values from calibrators with known methylation levels and methylation levels of the samples under study. This method aligns with the study objectives [31].

Melting curves were normalized using the Roche LightCycler 96 software. Difference plots were generated for each normalized melting curve relative to the baseline melting curve, which corresponded to the plot for the 100% methylated control sample. When multiple baseline samples were selected, the data from these curves were averaged and the resulting values were used as reference values for subtraction. After normalization of the difference plot, each curve was displayed as it appears when the AUC value for the baseline is subtracted.

The difference plots for calibrators and test samples were imported into Excel (Microsoft Office 2021) as a text file containing the fluorescence signal measurements data from each point along the temperature gradient. Further calculations were performed using the protocol proposed in Ref. [31] and available at https://dx.doi.org/10.17504/protocols.io.n2bvj6yjxlk5/v1.

Data were analyzed using the Statistica 10.0 software package. Frequencies of alleles and genotypes of the studied polymorphic variants were determined by direct counting and compared between groups using the χ^2 method and Fisher's exact test (for groups of <5). The Pearson correlation coefficient was used to test for correlation between DNMT1 mRNA expression level and blood homocysteine content. One-factor analysis of variance (ANOVA) was used to test for significant differences in *DNMT1* mRNA levels between the studied groups and to ascertain the impact of genotypes for the studied polymorphisms on *DNMT1* expression. Multivariate ANOVA was used to discern the collective influence of multiple independent variables. The data were tested for conformity to the normal distribution using the Kolmogorov–Smirnov test to assess if they met statistical assumptions.

Results

DNMT1 mRNA expression levels were significantly different across the experimental groups (ANOVA, $F = 17.5935$, $p = 0.0003$) (Fig. 2). Post-hoc analysis revealed a significant decrease in DNMT1 mRNA

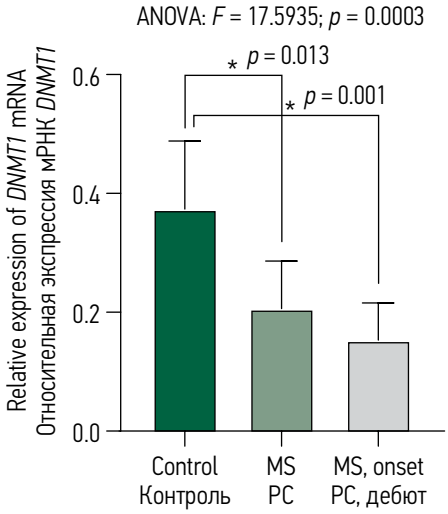

Fig. 2. The changes in the DNMT1 mRNA expression level in peripheral blood mononuclear cells in patients with multiple sclerosis (MS). Data are presented as means and the error of the mean; *significant differences between groups according to the results of post-hoc analysis, $p < 0.05$ (Tukey's test for unequal samples)

Рис. 2. Изменение уровня экспрессии мРНК *DNMT1* в периферических мононуклеарных клетках крови у пациентов с рассеянным склерозом (РС). Данные представлены в виде средних и ошибки среднего; *достоверные различия между группами по результатам апостериорного анализа, $p < 0,05$ (критерий Тьюки для неравных выборок)

levels in patients at MS onset ($p = 0.001$) and patients with a prolonged disease course ($p = 0.013$) relative to the control group.

These findings are consistent with the data of a previously published study that demonstrated a twofold decrease in the level of *DNMT1* mRNA expression in peripheral mononuclear blood cells of MS patients [19]. Furthermore, our study revealed that a significant suppression of *DNMT1* expression at the stage of disease onset.

There were no significant difference in degree of methylation of the promoter region of the *DNMT1* gene between samples obtained from healthy participants (control) and samples from MS patients. Values of percentage of methylation were 0%–2% in samples from the control group, and 0%–3.5% in samples from MS patients. However, there was a tendency toward a higher percentage of methylation in MS patients than in healthy participants (median 1.94 vs. 1.20). Figure 3 depicts the melting curves for calibration samples spanning 0%–100%. Furthermore, it includes a sample from cells of the HeLa tumor line with a methylation level of approximately 5% (used as a standard with an unknown percentage of methylation) and one of the tested samples.

There was a significant and strong positive correlation between the level of *DNMT1* mRNA expression and serum homocysteine content in patients

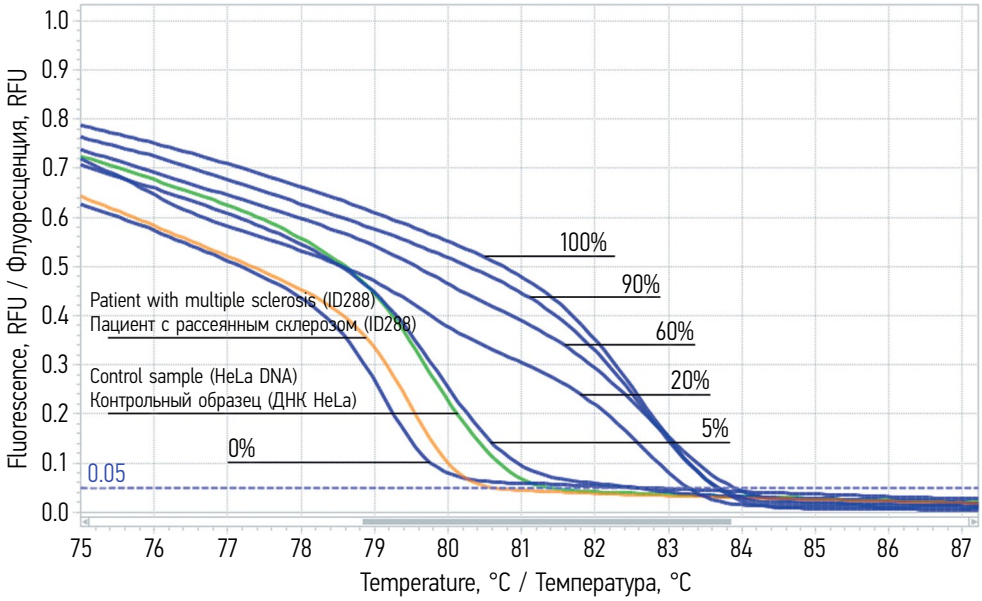

Fig. 3. Normalized melting curves for amplified *DNMT1* promoter fragments. Blue lines correspond to melting curves for calibration samples with known percentage of methylation; green line — melting curve for a sample obtained from the HeLa tumor cell line (calculated percentage of methylation 5.5%); orange line — sample obtained from peripheral blood mononuclear cells of a patient with multiple sclerosis (calculated percentage of methylation 1.15%)

Рис. 3. Нормализованные кривые плавления для амплифицированных фрагментов промотора *DNMT1*. Синие линии соответствуют кривым плавления для калибровочных образцов с известным процентом метилирования; зеленая линия — кривая плавления для образца, полученного из опухолевой клеточной линии HeLa (рассчитанный процент метилирования 5,5 %); оранжевая линия — кривая плавления образца, полученного из мононуклеарных клеток периферической крови пациента с рассеянным склерозом (рассчитанный процент метилирования 1,15 %)

Table 3 / Таблица 3

The data of correlation analysis to assess the relationship between the level of relative expression of *DNMT1* mRNA and the content of homocysteine in the blood
Данные корреляционного анализа для оценки взаимосвязи между уровнем относительной экспрессии мРНК *DNMT1* и содержанием гомоцистеина в крови

Group	Homocysteine / *DNMT1* mRNA
Control group ($n = 10$)	$r = 0.294$; $p = 0.442$
Multiple sclerosis, onset ($n = 10$)	$r = 0.388$; $p = 0.268$
Multiple sclerosis ($n = 10$)	$r = 0.733$; $p = 0.025$

N o t e: r is Pearson's correlation coefficient; p is the p value at 5% significance level.

with a disease duration of >1year (Table 3). No correlation between these parameters was observed in patients at the stage of MS onset and in the control group. In the group of patients with a disease duration of >1year, the disease duration was 4–15 years, with a median and interquartile range of 9 years and 7–12 years, respectively. There was no significant correlation between *DNMT1* mRNA expression level and disease duration in patients in this group ($r = -0.034$; $p = 0.931$). The observed patterns may reflect the interrelation of abnormalities in regulating methylation processes and changes in the functioning of folic acid metabolism and the methionine–homocysteine cycle in the development of the disease.

The influence of genotypes for *MTHFR, MTR*, and *MTRR* gene polymorphisms on *DNMT1* expression level was analyzed to determine the potential contribution of folate cycle gene polymorphisms to the mechanisms of epigenetic disturbances in MS. None of the four polymorphic variants studied (C677T, A1298C, A2756G, and A66G) demonstrated an isolated effect of genotype on *DNMT1* expression. However, a significant combined effect of genotypes at polymorphisms A2756G of the *MTR* gene and C677T of the *MTHFR* gene was observed on *DNMT1* mRNA expression level (ANOVA, $F = 4.516$; $p = 0.044$) (Fig. 4). A post-hoc analysis revealed significant differences in *DNMT1* mRNA expression levels between participants with the CC_{C677T}/AA_{A2756G} and those with $CC_{C677T}/AG,GG_{A2756G}$ genotypes ($p = 0.017$, Fisher's criterion). The CC_{C677T}/AA_{A2756G} genotype exhibited the lower *DNMT1* expression values.

A comparison of the distribution of genotypes for these polymorphic variants in the control group and in the group of MS patients (regardless of disease duration) revealed significant differences ($\chi^2 = 10.73$; $p = 0.014$). It turned out that $CC_{C677T}/AG,GG_{A2756G}$ genotype was significantly less frequent among MS patients than in the control group (15% and 35%, respectively). So, proportion of combinations of polymorphic variants with the highest values of relative *DNMT1* expression was reduced in MS patients. Additionally, the frequency of allele A for the A2756G polymorphism of the *MTR* gene was significantly higher in the MS patient group than in the control group ($\chi^2 = 4.655$; $p = 0.031$).

The observed patterns suggest that the risk of developing disorders associated with reduced *DNMT1* expression may be determined, at least partly, by genotype for the A2756G polymorphisms of the *MTR* gene and the C677T polymorphism of the *MTHFR* gene.

Discussion

MS is the most common demyelinating diseases. Whole genome analysis revealed significant differences in DNA methylation profiles between MS patients and healthy individuals [32]. These differences affect various genes involved in the regulation of immune cell activity, processes of oligodendrocyte maturation and differentiation, and formation of myelin structure [33–35]. Evidence of decreased expression (at the mRNA and protein levels) of DNMT1 and TET2, enzymes that control DNA methylation and demethylation is crucial for understanding

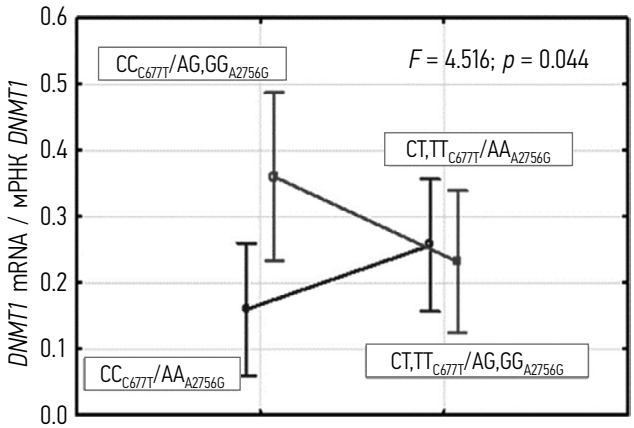

Fig. 4. The combined effect of genotypes for polymorphisms C677T of the *MTHFR* gene and A2756G of the *MTR* gene on the level of *DNMT1* expression in control group subjects and patients with multiple sclerosis

Рис. 4. Сочетанное влияние генотипов по полиморфизмам C677T гена *MTHFR* и A2756G гена *MTR* на уровень экспрессии *DNMT1* у участников контрольной группы и пациентов с рассеянным склерозом

the mechanisms of epigenetic dysregulation in MS. A twofold decrease in *DNMT1* mRNA expression in peripheral mononuclear cells from MS patients has been reported in a previous study [19]. Our data are consistent with those of previous studies and further demonstrate that changes in DNMT1 expression are characteristic of patients at the onset of MS.

Targeting the activity of methyltransferases with demethylating drugs is a possible approach for treating MS and other neurodegenerative diseases [36, 37]. Following the discovery of epigenetic changes specific to the pathogenesis of a number of neurodegenerative diseases, the possibility of targeted editing of methylation patterns is also being investigated [38]. Modification of folate metabolism, which is directly related to the regulation of methylation processes, can be considered an alternative and possibly a safer method. The folate cycle and methionine–homocysteine cycle are coupled metabolic pathways that ensure the formation of one-carbon fragments (methyl groups) necessary for the synthesis of DNA, amino acids, and methylation reactions [21]. Experimental studies reported that artificially induced deficiency of nutrients, primarily B vitamins, led to accumulation of homocysteine, a decrease in the rate of its conversion to methionine, deficiency of S-adenosine methionine, and changes in the activity of DNA methyltransferases [39, 40]. Furthermore, the presence of polymorphisms in genes encoding *MTR* and *MTHFR* has been reported to be associated with global or gene-specific changes in DNA methylation [41, 42]. The presence of polymorphisms in key genes of the folate cycle can affect the degree of methylation of the promoter region of the *DNMT1* gene in peripheral blood mononuclear cells [29]. Particularly, the study demonstrated an increase in the level of *DNMT1* promoter methylation in individuals carrying the minor allele G for the A2756G polymorphism of the *MTR* gene (rs1805087).

The findings of this study did not reveal an isolated effect of polymorphic variants of folate cycle genes on the level of *DNMT1* expression. However, we demonstrated the presence of a combined effect of genotypes for polymorphisms C677T of the *MTHFR* gene and A2756G of the *MTR* gene. When genotypes $CC_{C677T}/AG,GG_{A2756G}$ were combined, *DNMT1* expression values were the highest. These findings are consistent with those of previous studies, which indicate that the T allele of the C677T polymorphism is associated with DNA hypomethylation, and the G allele of the A2756G variant of the *MTR* gene is associated with hypermethylation [41]. Interestingly, the cumulative effect of polymorphic variants of folate cycle genes has been reported previously [43]. For further studies, it would be beneficial to verify the assumption that the genotype CC_{C677T}/AA_{A2756G}, which exhibits the greatest reduction in *DNMT1* expression levels, will be associated with a greater risk of disease development and/or progression.

Polymorphic variants, C677T and A2756G, are associated with an increased risk of hyperhomocysteinemia. The C–T substitution (C677T of the *MTHFR* gene) leads to the formation of a thermolabile form of the enzyme, to a decrease in its activity up to 70% in carriers of the CT genotype and up to 30% of the initial level in carriers of the TT genotype, which reduces the efficient formation of the active form of folic acid, 5-methyltetrahydrofolate, a coenzyme in the reaction of homocysteine remethylation [44]. The A2756G polymorphism impairs the function and stability of methionine synthase, encoded by the MTR gene, an enzyme that catalyzes the remethylation of homocysteine into methionine [45]. The correlation between *DNMT1* expression and the content of homocysteine, a marker of folate metabolism, revealed in this study and observed only in MS patients with long disease duration, lends support to the hypothesis that folic acid metabolism disturbances contribute to the dysregulation of epigenetic processes in MS. A weak significant inverse correlation between *DNMT1* promoter methylation level and homocysteine content was demonstrated previously in a cohort of healthy volunteers [29]. Considering that folate metabolism disturbances are potentially reversible, the investigating the association between epigenetic rearrangements and folic acid metabolism represents a promising avenue for identifying new markers and targets for MS therapy.

Conclusions

This study demonstrates, for the first time, that there is a reduction in *DNMT1* expression in MS patients at the disease onset stage. The correlation between *DNMT1* mRNA expression, blood homocysteine content, and genotype by *MTHFR* and *MTR* gene polymorphisms supports the hypothesis that changes in the metabolism of one-carbon fragments caused by the presence of polymorphic variants of folate cycle genes are involved in the pathogenesis of MS and may contribute to impaired epigenetic regulation.

Additional information

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Compliance with ethical standards. The study was approved by the Local Ethics Committee of the Federal State Budgetary Institution “IEM” (protocol No. 3/23 of September 20, 2023). Before the study, voluntary informed consent was obtained from all subjects whose data are presented in the publication.

Conflict of interest. The authors declare no conflict of interest.

Authors' contribution. All authors made significant contributions to the conception, conduct of the study and preparation of the article, and read and approved the final version before publication. The largest contribution is distributed as follows: *E.A. Tsymbalova* — conducting experiments, processing results; *E.A. Chernyavskaya* — conducting experiments, selecting literature, processing results; *D.E. Ryzhkova* — conducting experiments, describing, processing results; *G.N. Bisaga, I.N. Abdurasulova* — discussion of the results; *V.I. Lyudyno* — concept and management of the work, processing of results, writing the text.

Дополнительная информация

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