DISEASE-ASSOCIATED HLA-DR POLYMORPHISM, CLINICAL AND IMMUNOLOGICAL CHARACTERISTICS OF MULTIPLE SCLEROSIS PATIENTS IN THE NORTHEASTERN REGION OF UKRAINE

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The development of multiple sclerosis is the result of complex interactions between environmental factors, genetic factors that determine individual disease susceptibility, and immunological and physiological characteristics of the patient [1, 2]. Multiple sclerosis treatment requires obtaining information about the main pathological processes, therefore, the evaluation of biochemical, immunological, and genetic markers of such processes, and not just clinical indicators, can become the basis of improved approaches for diagnosis and monitoring of the disease [3-6]. Polymorphism of the disease-associated genes is considered as one of the key factors in multiple sclerosis pathogenesis, capable of influencing the risk of development, clinical manifestations and nature of the course of the disease, treatment response [7]. The HLA genes polymorphism was found to be the most important and playing an essential role in the development of autoimmune diseases. For example, the presence of the polymorphic version of HLA-DRB1*15:01 gives a threefold increase in the risk of multiple sclerosis, and in the case of monozigency, the disease susceptibility increases by 6-7 times [7-9]. There are data on the population characteristics of these types of polymorphisms, which require conducting relevant research in Ukraine and its regions to identify relevant genetic markers [9, 10]. **The aim of the study** was to provide a comparative clinical and immunological characteristic of multiple sclerosis patients in the northeastern region of Ukraine, depending on the presence of the disease-associated HLA-DR polymorphism.

Materials and methods

39 patients with multiple sclerosis, inhabitants of Kharkiv and Kharkiv region, were examined, of which 7 men and 32 women of medium age of 33.7 ± 7.7 and 42.1 ± 11.9 years, respectively; control group was consisted of 27 practically healthy persons of both sexes with an average age of 30.1 ± 8.2 years. Patients were on outpatient and inpatient treatment at the Institute of Neurology, Psychiatry and Narcology of the National Academy of Medical Sciences of Ukraine, the study was carried out on the basis of the Laboratory of Clinical Immunology and Allergology, Mechnikov Institute of Microbiology and Immunology of the National Academy of Medical Sciences of Ukraine. The inclusion criteria were 1) the presence of a verified diagnosis of multiple sclerosis (code G35 according to ICD-10), the formulation of which was made in accordance with the order of the Ministry of Health of Ukraine № 487 dated 17.08.2007, 2) the lack of disease modifying therapies for at least six months before the survey began. All patients participated in the study gave voluntary written agreement.

Clinical characteristics of multiple sclerosis patients included the determining the form (sporadic or familial) and the actual type of the disease, its duration, and disability assessment based on the EDSS scale. The rate of the disease progression was determined as the ratio of the EDSS score to the duration of the disease.

Biological material was blood and buccal epithelium samples from multiple sclerosis patients and practically healthy people. In addition to assessing the clinical status of the patients, design of the study also included evaluation of the systemic immunity indicators, levels of nonspecific and antinuclear antibodies in the serum, and analysis of HLA polymorphism, in particular, detection of the HLA-DRB1*1501-DQB1*0602 (HLA-DR15) haplotype for its specific marker SNP rs9271366. Isolation of high molecular DNA (40.000 – 50.000 bp, OD 260/280 nm) was performed on a magnetically sensitive sorbent using the NeoPrep100 DNA Magnet kit (NeoGene, Ukraine). SNP polymorphism rs9271366 A/G was typed by the allele-specific amplification method with subsequent electrophoretic detection of the results. Primer selection was performed using the method [11] adding a mismatch in the third position at the 3'ends. The primers MS92AF 5`-CACGTAATATAA-ATGGTTGCAAAGGA-3`, MS92GF 5`-CACGTAATATAAATGGTTGCA-AAGGG-3` and MS92R5` AACCCTGATGTAACAGA(C/T)CTCTA-3` (Eurofins Genomics), as well as Taq-mut polymerase (Liteh, Russian Federation), were used in the study. The amplification was performed on the multichannel amplifier Tercyc (Russian Federation). Amplification mode: denaturation at 96°C for 3 minutes and 35 cycles that included denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, and synthesis at 72°C for 30 seconds. The length of the amplicon was 233 bp. Electrophoresis was performed in 2% agarose gel in TAE buffer. Electrophoregram analysis was carried out on transilluminator UVT 1 (Biocom, Russian Federation).

Serum IgM, IgG, IgA levels were evaluated by ELISA using a test system by NPL Granum (Ukraine) and the immunoassay analyzer Stat-Fax 303 (USA). The determination of peripheral blood lymphocytes subpopulations was carried out using the test systems "Anti-CD3", "Anti-CD4", "Anti-CD8", "Anti-CD16", "Anti-CD22" (NPL Granum, Ukraine). Circulating immune complexes (CIC) in serum were evaluated by selective precipitation of antigen complexes with 3.5% PEG-6000 solution (AppliChem GmbH, Germany). The optical density of precipitate was measured spectrophotometrically on a Stat-Fax 303 analyzer at 450 nm. The content of lymphocytotoxic autoantibodies

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(LCTA) was determined by a lymphocytotoxic macrotest. The complementary activity of the blood serum was evaluated by means of 50% of sheep erythrocytes hemolysis in the presence of homologous antibodies.

The statistical treatment was performed using Statistica 11.0 (StatSoft, Inc). To determine the reliability of the differences between the indexes in the studied samples, the non-parametric Mann-Whitney's criterion was used, while the Student's T-criterion was used for the normal distribution.

Results and discussion

At the stage of clinical examination of multiple sclerosis patients, 25 patients (64.1%) were diagnosed with relapsing-remitting type of multiple sclerosis (RRMS), 4 patients (10.3%) with primary progressive type (PPMS), and another 10 persons (25,6%) with secondary progressive type (SPMS). The familial form of the disease was established in 11 cases (28.2%), in 28 cases (71.8%) the form of the disease was classified as sporadic. The average disease duration of the examined persons was 10.16 ± 9.57 years. The disability score assessed according to the EDSS scale was 3.54 ± 1.67 points; the rate of progression of the disease was 0.93 ± 1.26 and 0.83 ± 0.85 points, respectively.

The next stage of the study was to establish the presence of the "risk" haplotype HLA-DRB1*1501-DQB1*0602 in patients by means of the one of its specific markers (tag SNP), SNP rs9271366. According to [12], the detection of HLA-DRB1*1501 by typing SNP rs9271366 has a high sensitivity and specificity of more than 97%. The technique we used allowed us to clearly differentiate the allele of the given locus. Analysis of the HLA polymorphism indicated that the disease-associated (minor) allele G SNP rs9271366 was present in 43.6% of the subjects examined (heterozygous), and another 56.4% of the patients were homozygous by the major allele A. According to the presence of alleles A and G of SNP rs9271366, multiple sclerosis patients were divided into groups G+ (heterozygous, n=17) and G- (homozygous for allele A, n=22). All persons in the control group were homozygous for the allele A.

Analysis of the data allowed concluding that the form of the disease, disability score and the rate of progression in multiple sclerosis patients had no association with the SNP rs9271366 minor allele. The average EDSS score was 3.45 ± 1.95 points in G+ patients and 3.65 ± 1.26 points in G- patients and the rate of disease progression was 0.83 ± 0.85 points and 0.93 ± 1.26 points, respectively.

The study of cellular immunity indicators (table 1) revealed a decrease in relative number of CD4+ and CD8+ lymphocytes in multiple sclerosis patients compared to that in control group, and it was more pronounced in subjects with the presence of the SNP rs9271366 minor allele. The relative content of CD4 + cells was 28.00 ± 3.45% in heterozygous patients, 30.94 ± 4.42% in patients homozygous for allele A, in the control group 42.3 ± 1.8% ($p<0.05$), and the CD8+ cells content was 18,55 ± 4.08%, 20.65 ± 3.52% and 29.4 ± 2.2%, respectively ($p<0.05$). At the same time, the reduction in the relative number of CD4+ lymphocytes was observed in 90.9% of G+ patients, and in 64.7% cases in G- patients ($p<0.05$). Decreased CD8+ lymphocyte content was also more common in patients with G+ (77.3% of cases) than in G- (41.2% of cases), $p<0.05$.

Table 1 - Cellular immunity in patients with multiple sclerosis, M±σ

Indicators	Units	MS patients			Control group
		Overall	G- group	G+ group	
Leukocytes	cells x10^9/l	6,5 ± 2,4	6,6 ± 1,9	6,4 ± 2,7	6,7 ± 1,6
Lymphocytes	%	29,7 ± 9,9	30,2 ± 7,6	29,4 ± 11,4	28,9 ± 5,7
	cells x10^9/l	1,8 ± 0,6	2,0 ± 0,6	1,7 ± 0,6	1,9 ± 0,3
CD3+	%	51,7 ± 8,9	53,1 ± 9,0	50,1 ± 7,0	62,7 ± 4,8
	cells x10^9/l	1,0 ± 0,4	1,1 ± 0,4	0,9 ± 0,3	1,2 ± 0,2
CD4+	%	29,9 ± 4,9*	30,9 ± 4,4*	28,0 ± 3,5*	42,3 ± 1,8
	cells x10^9/l	0,5 ± 0,2	0,6 ± 0,2	0,5 ± 0,2	0,8 ± 0,2
CD8+	%	19,9 ± 4,4*	20,7 ± 3,5*	18,6 ± 4,1*	29,4 ± 2,2
	cells x10^9/l	0,4 ± 0,2	0,4 ± 0,2	0,3 ± 0,2	0,6 ± 0,1
CD16+	%	17,9 ± 2,5	18,1 ± 2,2	17,6 ± 2,5	18,5 ± 1,7
	cells x10^9/l	0,3 ± 0,1	0,3 ± 0,1	0,3 ± 0,1	0,4 ± 0,1
CD22+	%	17,4 ± 3,9	17,1 ± 4,5	18,1 ± 3,2	22,1 ± 2,6
	cells x10^9/l	0,3 ± 0,1	0,4 ± 0,1	0,3 ± 0,1	0,4 ± 0,1

* ($p < 0,05$) compared to the control group.

Acute inflammatory process was determined in 38.5% of patients with multiple sclerosis, as evidenced by an elevated IgM level in 20.5% of cases, as well as an increase in complement activity in 62.5% of subjects (table 2). The levels of LCTA in patients with multiple sclerosis were significantly higher compared to the control group (18.33 ± 3.67% of subjects and 5.82 ± 3.12% of subjects, respectively, $p<0.05$), and the levels of circulating immune complexes were elevated in 10.3% of patients who participated in the study.

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Table 2 - Humoral immunity in patients with multiple sclerosis, M±σ

Indicators	Units	MS patients			Control group
		Overall	G- group	G+ group	
IgA	g/l	2,03 ± 0,46	1,94 ± 0,4	2,13 ± 0,49	1,8 ± 0,39
IgG	g/l	12,69 ± 2,43	12,43 ± 1,87	12,98 ± 2,42	10,2 ± 1,12
IgM	g/l	1,46 ± 0,42	1,47 ± 0,44	1,44 ± 0,39	1,15 ± 0,27
CIC	conv. units	77,82 ± 28,22	75,12 ± 19,52	82,0 ± 28,59	84,55 ± 14,1
LCTA	%	17,33 ± 4,58*	18,18 ± 4,42*	18,45 ± 3,07*	5,82 ± 3,12
Complement activity	hem. units	57,24 ± 12,08	62,06 ± 9,02	54,52 ± 12,83	44,21 ± 7,64

* ($p < 0,05$) compared to the control group.

The study of the levels of antinuclear antibodies in multiple sclerosis patients revealed a diagnostically significant levels of antibodies to native DNA found in 92.3% of the cases, including all G + patients and 83.3% of G- patients (table 3).

Table 3 – Cases of diagnostically significant levels of antinuclear antibodies depending on SNP rs9271366, %

Indicators	MS patients		Control group
	G- group	G+ group	
Abs to native DNA	83,3	100	-
Abs to denaturated DNA	16,7	14,3	-
Abs to formalinized DNA	16,7	28,6	3,7

In this case, 16.7% of patients homozygous for allele A and 14.3% heterozygous patients were also positive for the presence of antibodies to denatured DNA. Similar results were also obtained concerning the presence of antibodies to formalinized DNA - 16.7% positive results in G- patients and 28.6% in G+ patients ($p<0.05$). At the same time, elevated levels of antibodies to native, denatured, and formalinized DNA were determined predominantly in patients with SPMS and PPMS (15.4% of the number of examined patients).

Conclusion

Among the surveyed multiple sclerosis patients, inhabitants of Kharkiv and Kharkiv region, the presence of the SNP rs9271366 minor allele G was established in 43.6% (heterozygous, haplotype AG), while 56.4% of the patients were homozygous for allele A (haplotype AA). The comparative analysis of clinical indicators and genetic data of examined patients indicated that the type of the disease, EDSS score, and the rate of multiple sclerosis progression did not have association with the allele G SNP rs9271366. Also, in patients with familial and sporadic forms of the disease, there were no significant differences in clinical indicators and immune status. In patients with multiple sclerosis, the inhibition of the cellular immunity was detected, as evidenced by a significant decrease in the relative number of CD4+ and CD8+ lymphocytes, more pronounced in patients with the presence of the SNP rs9271366 minor allele. The levels of lymphocytotoxic autoantibodies as well as of antinuclear antibodies in patients were significantly higher than that in the control group. Antibodies to native DNA were detected in 92.3% of patients. At the same time, the raised levels of antibodies to the native, denaturized and formalinized DNA were determined predominantly in patients with SPMS and PPMS. The elevated levels of antinuclear antibodies may indicate an unfavorable course of the disease or its transition to the active phase.

Thus, in the surveyed multiple sclerosis patients from the northeastern region of Ukraine, the prevalence of SNP rs9271366 and its association with a decrease in the relative number of CD4+ and CD8+ cells against the background of elevated levels of antinuclear and lymphocytotoxic antibodies were detected.

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The development of multiple sclerosis is the result of complex interactions between environmental factors, genetic factors that determine individual disease susceptibility, and immunological and physiological characteristics of the patient. multiple sclerosis treatment requires obtaining information about the main pathological processes, therefore, the evaluation of biochemical, immunological, and genetic markers of such processes, and not just clinical indicators, can become the basis of improved approaches for diagnosis and monitoring of the disease. Polymorphism of the disease-associated genes is considered as one of the key factors in multiple sclerosis pathogenesis, capable of influencing the risk of development, clinical manifestations and nature of the course of the disease, treatment response. The HLA genes polymorphism was found to be the most important and playing an essential role in the development of autoimmune diseases. There are data on the population characteristics of these types of polymorphisms, which require conducting relevant research in Ukraine and its regions to identify relevant genetic markers. **The aim of the study** was to provide a comparative clinical and immunological characteristic of multiple sclerosis patients in the northeastern region of Ukraine, depending on the presence of the disease-associated HLA-DR polymorphism. **Materials and methods.** 39 patients with multiple sclerosis, inhabitants of Kharkiv and Kharkiv region, were examined, of which 7 men and 32 women of medium age of 33.7 ± 7.7 and 42.1 ± 11.9 years, respectively; control group was consisted of 27 practically healthy persons of both sexes with an average age of 30.1 ± 8.2 years. Clinical characteristics of multiple sclerosis patients included the determining the form and the actual type of the disease, its duration, and disability assessment based on the EDSS scale. The rate of the disease progression was determined as the ratio of the EDSS score to the duration of the disease. Biological material was blood and buccal epithelium samples from multiple sclerosis patients and practically healthy people. In addition to assessing the clinical status of the patients, design of the study also included evaluation of the systemic immunity indicators, levels of nonspecific and antinuclear antibodies in the serum, and analysis of HLA polymorphism, in particular, detection of the HLA-DR15 haplotype for its specific marker SNP rs9271366. Isolation of high molecular DNA was performed on a magnetically sensitive sorbent using the NeoPrep100 DNA Magnet kit (NeoGene, Ukraine). SNP polymorphism rs9271366 A/G was typed by the allele-specific amplification method with subsequent electrophoretic detection of the results. Primer selection was performed using the method by Liu Jing et al. (2012) adding a mismatch in the third position at the 3'ends. The primers MS92AF 5`-CACGTAATATAA-ATGGTTGCAAAGGA-3`, MS92GF 5`-CACGTAATATAAATGGTTGCA-AAGGG-3` and MS92R5` AACCCTGATGTAACAGA(C/T)CTCTA-3` (Eurofins Genomics), as well as Taq-mut polymerase (Liteh, Russian Federation), were used in the study. The amplification was performed on the multichannel amplifier Tercyc (Russian Federation). Amplification mode: denaturation at 96°C for 3 minutes and 35 cycles that included denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, and synthesis at 72°C for 30 seconds. The length of the amplicon was 233 bp. Electrophoresis was performed in 2% agarose gel in TAE buffer. Electrophoregram analysis was carried out on transilluminator UVT 1 (Biocom, Russian Federation). Serum IgM, IgG, IgA levels were evaluated by ELISA using a test system by NPL Granum (Ukraine) and the immunoassay analyzer Stat-Fax 303 (USA). The determination of peripheral blood lymphocytes subpopulations was carried out using the test systems by NPL Granum, Ukraine in accordance

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with the manufacturer's instructions. Circulating immune complexes in serum were evaluated by selective precipitation of antigen complexes with 3.5% PEG-6000 solution (AppliChem GmbH, Germany). The content of lymphocytotoxic autoantibodies was determined by a lymphocytotoxic macrotest. The complementary activity of the blood serum was evaluated by means of 50% of sheep erythrocytes hemolysis in the presence of homologous antibodies. The statistical treatment was performed using STATISTICA 11.0 (StatSoft, Inc). To determine the reliability of the differences between the indexes in the studied samples, the non-parametric Mann-Whitney's criterion was used, while the Student's T-criterion was used for the normal distribution. **Results.** At the stage of clinical examination of multiple sclerosis patients, 25 patients (64.1%) were diagnosed with RRMS, 4 patients (10.3%) with PPMS, and another 10 persons (25,6%) with SPMS. The familial form of the disease was established in 11 cases (28.2%), in 28 cases (71.8%) the form of the disease was classified as sporadic. The average disease duration of the examined persons was 10.16 ± 9.57 years. The disability score assessed according to the EDSS scale was 3.54 ± 1.67 points; the rate of progression of the disease was 0.93 ± 1.26 and 0.83 ± 0.85 points, respectively. Analysis of the HLA polymorphism indicated that the disease-associated (minor) allele G SNP rs9271366 was present in 43.6% of the subjects examined (haplotype AG, G+ group), and another 56.4% of the patients were homozygous by the major allele A (haplotype AA, G- group). The form of the disease, the EDSS score, and the rate of the disease progression in the examined patients did not have association with the allele G SNP rs9271366. Patients with familial and sporadic forms of multiple sclerosis did not have significant differences in immune status and clinical indicators. The average EDSS score was 3.45 ± 1.95 points in G+ patients and 3.65 ± 1.26 points in G- patients and the rate of disease progression was 0.83 ± 0.85 points and 0.93 ± 1.26 points, respectively. The study of cellular immunity indicators revealed a decrease in relative number of CD4+ and CD8+ lymphocytes in multiple sclerosis patients compared to that in control group, and it was more pronounced in subjects with the presence of the SNP rs9271366 minor allele. The relative content of CD4 + cells was 28.00 ± 3.45% in heterozygous patients, 30.94 ± 4.42% in patients homozygous for allele A, in the control group 42.3 ± 1.8% ($p<0.05$), and the CD8+ cells content was 18,55 ± 4.08%, 20.65 ± 3.52% and 29.4 ± 2.2%, respectively ($p<0.05$). At the same time, the reduction in the relative number of CD4+ lymphocytes was observed in 90.9% of G+ patients, and in 64.7% cases in G- patients ($p<0.05$). Decreased CD8+ lymphocyte content was also more common in patients with G+ (77.3% of cases) than in G- (41.2% of cases), $p<0.05$. Acute inflammatory process was determined in 38.5% of patients with multiple sclerosis, as evidenced by an elevated IgM level in 20.5% of cases, as well as an increase in complement activity in 62.5% of subjects. The levels of lymphocytotoxic autoantibodies in patients with multiple sclerosis were significantly higher compared to the control group (18.33 ± 3.67% of subjects and 5.82 ± 3.12% of subjects, respectively, $p<0.05$), and the levels of circulating immune complexes were elevated in 10.3% of patients. The study of the levels of antinuclear antibodies in multiple sclerosis patients revealed a diagnostically significant levels of antibodies to native DNA found in 92.3% of the cases, including all G + patients and 83.3% of G- patients. In this case, 16.7% of patients homozygous for allele A and 14.3% heterozygous patients were also positive for the presence of antibodies to denatured DNA. Similar results were also obtained concerning the presence of antibodies to formalinized DNA - 16.7% positive results in G- patients and 28.6% in G+ patients ($p<0.05$). At the same time, elevated levels of antibodies to native, denatured, and formalinized DNA were determined predominantly in patients with SPMS and PPMS (15.4% of the number of examined patients). The elevated levels of antinuclear antibodies may indicate an unfavorable course of the disease or its transition to the active phase. **Conclusion.** In the surveyed multiple sclerosis patients from the northeastern region of Ukraine, the prevalence of SNP rs9271366 and its association with a decrease in the relative number of CD4+ and CD8+ cells against the background of elevated levels of antinuclear and lymphocytotoxic antibodies were detected.

Keywords: multiple sclerosis, disease-associated polymorphism of genes, immunopathogenesis.

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