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Elevated serum homocitrulline levels in patients with multiple sclerosis and its relationship with disease activity

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Abstract

Aim: Multiple sclerosis (MS) is a demyelinating disease of central nervous system. Myelin basic protein (MBP) is the major protein in the structure of the myelin sheath, and the abnormal autoimmune response to MBP is related to the demiyelinization in MS. This autoimmune response is thought to be related to extensive post-translational modifications that may occur in the primary structure of MBP. Considering the role of post-translational modifications of MBP in the pathogenesis of MS, this study aimed to investigate the role of carbamylation in the pathogenesis of MS by measuring Hcit levels in patients with MS.

Materials and Methods: This study included 80 patients with MS according to McDonald criteria by clinicians, and 60 healthy volunteers. Patients were divided into 2 groups as relapsing-remitting multiple sclerosis (RRMS) (n=44) and secondary progressive MS (SPMS) (n=36) according to Expanded Disability Status Scale (EDSS) score evaluated by clinicians. Serum homocitrulline and lysine levels were measured with validated tandem mass spectrometric method.

Results: Serum Hcit levels in patients with MS were statistically significantly higher than the healthy controls. Comparison of MS subgroups according to Hcit levels showed that serum Hcit levels were higher in patients with SPMS than in patients with RRMS. Serum Hcit levels were positively correlated with EDSS and disease duration.

Conclusion: Serum Hcit levels were significantly elevated in patients with MS. Moreover, there was a correlation between serum Hcit levels and disease activity and duration of disease.

Introduction

Multiple sclerosis is an autoinflammatory, chronic, demyalinating, neurodegenerative disease affecting the central nervous system [1]. MS is related to immune dysregulation resulting in infiltration of immune system components into the central nervous system, damage to the blood-brain barrier, demyelination, axonal damage, and neurodegeneration [2]. MS is a common neurodegenerative disease and approximately 2.5 million individuals worldwide suffer from MS. The age of onset is between 20-50 years and it is about 3 times more common in women than men [3]. The myelin sheath is a lipid and protein-containing protective sheath that covers most axons in the brain and spinal cord, plays a role in facilitating neurotransmission and in trophic support. Myelin basic protein (MBP) is one of the main proteins of the myelin sheath and plays a role in maintaining the stability of the myelin sheath [4]. The mechanisms involved in MS pathogenesis are still unclear, but demyelination, characterized by a T cell-mediated inflammatory response to MBP, plays a key role in the pathogenesis of MS [5]. It is thought that MBP may trigger a neurodegeneration in MS that makes myelin sheaths prone to degradation. MBP is one of the proteins that can be citrullinate under normal physiological conditions, but hypercitrullination of this protein has been demonstrated in MS patients and it has been reported that the MBP isoforms formed react differently with T cells. Therefore, it is thought that posttranslational modifications that may occur in the primary structure of MBP may contribute to the autoantigen presentation that initiates the autoimmune response and causes myelin damage in MS patients [6, 7]. Homocitrulline (Hcit) is a structural homologue of citrulline and may be found in proteins and

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peptides as a posttranslational modification product. Hcit formation is known as carbamylation and Hcit is formed spontaneous or enzymatically as a result of the reaction between lysine residues in proteins and isocyanate [8]. Isocyanic acid, which is required for the carbamylation of lysine residues, can be produced in two ways: urea deamination and oxidation of thiocyanate by myeloperoxidase (MPO). Under normal conditions, serum urea and isocyanic acid levels are in equilibrium. However, in cases characterized by renal failure, isocyanic acid levels increase in parallel with blood urea levels. Apart from spontaneous carbamylation, there is another enzyme-mediated mechanism of carbamylation. MPO is a heme-containing enzyme presented in inflammatory cells such as neutrophils, monocytes, and macrophages, and it catalyzes the oxidation of thiocyanate to cyanate in the presence of hydrogen peroxide [9]. In this reaction, MPO mediates the conversion of thiocyanate from smoking or diet to active cyanate, thus this reaction indicates the relationship between inflammation, smoking, diet, and carbamylation [8]. Carbamylation plays an important role pathogenesis of different diseases by changing the charges, structures and functions of proteins [10]. It has been shown that the presence of anticarbamylated protein (anti-CarP) antibodies in addition to citrullinated protein antigens (ACPA) in patients with rheumatoid arthritis. Therefore, recent findings indicate that Hcit-containing proteins may be involved in the pathogenesis of autoimmune diseases by gaining autoantigenic properties [11]. Considering the role of posttranslational modifications of MBP in the pathogenesis of MS, the aim of this study was to investigate the role of carbamylation in the pathogenesis of MS by measuring Hcit levels in patients with MS.

Materials and Methods

Study design

Participants

Eighty patients who applied to Selcuk University Faculty of Medicine Neurology outpatient clinic and were diagnosed with MS according to McDonald criteria by clinicians, and 60 healthy volunteers without any chronic disease were enrolled [12].

Exclusion criteria for the patient and control groups were as follows:

1. Cardiovascular diseases
2. Diabetes mellitus
3. Smoking or alcohol usage
4. Renal disorders
5. Liver disease
6. Other systemic inflammatory or autoimmune diseases
7. Infectious diseases
8. Endocrine disorders
9. Other neurological disorders.

There were 118 MS patients who were registered in the Neurology Polyclinic of Selcuk University Faculty of Medicine and were followed up regularly. However, the following patients were excluded from the study: 18 patients with cardiovascular disease and diabetes, 2 patients with kidney failure, 2 patients with fatty liver disease, 8 patients with other autoimmune and infectious diseases, and 8 patients who did not give consent. Thus, the sample size of patients group was determined as 80. After the exclusion criteria were applied, the control group was selected as age and sex-matched healthy individuals with the patient group. Patients were divided into 2 groups as relapsing-remitting multiple sclerosis (RRMS) (n=44) and secondary progressive MS (SPMS) (n=36) according to Expanded Disability Status Scale (EDSS) score evaluated by clinicians.

The blood samples of subjects were drawn into the anticoagulant-free (4 mL) and EDTA tubes (2 mL). Blood in anticoagulant-free tubes was centrifuged at 3500 g during 10 minutes and separated serum samples kept at -80ºC until analysis of homocitrulline and lysine. Written informed consent was obtained from participants. This study was approved by Selcuk University Faculty of Medicine Ethics Committee local ethics committee and this study was conducted in accordance with the Declaration of Helsinki.

Instrumentation

The analytes were detected with API 3200 triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex) in positive electrospray ionization mode. Shimadzu HPLC system (Kyoto, Japan) and Phenomenex C18 HPLC column (50 mm x 4.6 mm) were used for chromatographic analysis. To provide the elution of the analytes, a mixture of 0.1% formic acid and acetonitrile (80:20; v: v%) was applied isocratically as the mobile phase. Total flow rate, run time and column oven temperature were 1 mL/min, 5 min and 40 °C, respectively. The Q1 to Q3 ion transitions were 246.0/127.0, 203.0/84.0, 236.0/74.0 and 203.0/84.0 for Hcit, lysine, d4-L-citrulline and d8-L-lysine, respectively. Method optimization parameters including ionspray voltage, temperature, ion source (GS1) and ion source (GS2), curtain, collision gas values were 5000 V, 400 OC, 40, 60, 20, 5 psi, respectively. The interassay imprecision values of the method for homocitrulline and lysine were less than 9.1%. The mean recovery value was 95.8% for both analytes.

Sample preparation

Hcit and lysine concentrations were quantitated via modified method reported by Dietzen et al. [13]. Briefly; 50 µl of d4-L-citrulline, 50 µl of d8-L-lysine and 850 µl of methanol were added to sample and the mixture was vortexed during 30 seconds. After 10 minutes of incubation at room temperature, the samples were centrifuged at 2000 g for 15 minutes and the supernatants were drawn into clean reaction tubes. They were evaporated under nitrogen gas at 65 °C, then 200 µl of 3N HCl / n-butanol solution was added and sealed tubes were kept at 65 °C during 30 minutes for derivatization. After incubation, the evaporation process was repeated. The dried samples were dissolved in 200 µL of mobile phase mixture and 40 µL was enjected into LC-MS/MS system.

Laboratory measurements

White blood cell count (WBC), neutrophil (NEU), monocyte (MONO), lymphocyte (LYM), hemoglobin (HGB), platelet (PLT) counts, mean platelet volume (MPV) and red cell distribution width (RDW) levels were measured with Beckman Coulter LH 780 analyzer (Beckman Coulter, Miami, FL, USA). Serum creatinine (CREA), urea, triglyceride (TG), glucose (GLU), total cholesterol (TC), high-density lipoprotein (HDL-C), low-density lipoprotein (LDL-C), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured with Beckman-Coulter AU 5800 (Beckman Coulter, Brea, USA).

Statistical analysis

Statistical analyses were performed using SPSS software version 22.0 (IBM, Armonk, NY, USA). The distribution of data was checked with the One-Sample Kolmogorov-Smirnov test. Parametric and non-parametric parameters were compared with Student's t and Mann-Whitney U tests, respectively. One-way ANOVA analysis (post-hoc analysis with LSD or Tamhane's T2 tests) and Kruskal – Wallis test (post-hoc analysis Mann-Whitney U) were performed for comparison of more than two groups. Bonferroni correction was applied while performing Mann-Whitney U test for pairwise comparisons after Kruskal-Wallis test and $p<0.017$ accepted as statistically significant. Correlation was evaluated with Spearman correlation test. $p<0.05$ was accepted as statistically significant except that Bonferroni correction.

Results

The mean ages of RRMS, SPMS and control groups were 36.73±6.87, 39.34±5.98 and 37.62 ±5.52 years, respectively (p=0.528). Basic demographic and clinical parameters of participants were shown in Table 1.

Serum Hcit levels were statistically significantly higher in the MS group than the control group ($p<0.001$). In addition, RDW (p=0.003), MPV (p=0.040), NEU (p=0.025), PLR ($p<0.001$), NLR ($p<0.001$), MLR ($p<0.001$) levels were statistically significantly higher in MS patients, while LYM ($p<0.001$) levels were low (Table 2).

Statistical results of MS subgroups were evaluated with Bonferroni correction. Comparison of MS subgroups according to Hcit levels showed that serum Hcit levels were higher in patients with SPMS than in patients with RRMS while the PLT levels were higher in the RRMS group than in the SPMS group ($p<0.017$). PLR, NLR, MLR, RDW, PLT and serum Hcit levels were statistically significantly higher in the RRMS group than in the control group while LYM levels were lower ($p<0.017$). Similarly, PLR, NLR, MLR, and serum Hcit levels were statistically significantly higher in the SPMS group than in the control group while LYM levels were lower ($p<0.017$). Table 3 was included to show the comparison between the MS subgroups and the control group.

Serum Hcit levels were positively correlated with EDSS (r=0.294, p=0.008) and disease duration (r=0.312, p=0.005). There was a positive correlation between serum Hcit levels with inflammation markers RDW (r=0.318, $p<0.001$), MPV (r=0.331, $p<0.001$), PLT (r=0.174, p=0.043), NEU (r=0.311, $p<0.001$), PLR (r=0.226, p=0.008), NLR (r=0.313, $p<0.001$), MLR (r=0.240, p=0.005) levels and there was a negative correlation with LYM levels (r= -0.168, p=0.049). In addition serum Hcit levels were positively correlated with serum urea levels (r=0.251, p=0.003).

Discussion

MBP is the primary myelin protein accounting for approximately 35% of the total myelin protein and is a potent candidate autoantigen [14]. It is considered that MBP involved in the pathogenesis of demyelinating diseases. Extensive post translational modification of MBP has been reported to involved in the pathogenesis of multiple sclerosis [15]. In humans, MBP is a 170 amino acid protein and contains 19 arginyl and 12 lysyl residues [16]. However, its primary structure undergoes extensive post-translational modification by phosphorylation, methylation, citrullination of arginyl residues, deamidation of glutaminyl residues, and N-terminal acetylation [15]. It has been reported that approximately 20% of MBP in healthy individuals contains citrulline residues, while approximately 45% of MPB in the central nervous system of MS patients has been shown to contain citrulline residues [17, 18]. In patients with fulminant MS, it has been shown that 80-90% of MBP contains citrulline residues [19]. In addition, previous studies have demonstrated that elevated serum citrulline concentrations in patients with MS [4, 20]. Excessive post-translational modification of MBP by mechanisms such as methylation, phosphorylation and citrullination can trigger conformational and structural changes in MBP and affect the interaction of MBP with myelin membranes and other proteins. Therefore, post-translational modifications in MBP are thought to be one of the primary factors responsible for the autoantigenicity of MPB and the development of MS in mammals [15].

Carbamylation is one of the post-translational mechanisms that occurs by adding isocyanic acid formed from thiocyanate via MPO or urea to the free amino groups of proteins [21]. Recent studies have shown that protein carbamylation related to the pathogenesis of various diseases such as inflammatory, autoimmune, cardiovascular, renal diseases and physiological conditions such as aging [22-26]. Hcit, which is formed by the binding of isocyanic acid to the ε-NH2 group of lysine residues in proteins, is the most characteristic carbamylation product [27]. Elevated serum Hcit concentrations has been reported in patients with coronary artery disease and chronic renal failure [28, 29], and Shi et al. showed the presence of auotoantibodies to carbamylated proteins in both ACPA positive and ACPA negative RA patients. Moreover, they reported that the anti-CarP positive group had more erosive forms of RA. Therefore, similar to citrulline, the immunogenicity of Hcit has been demonstrated in patients with RA [11]. Therefore, considering the role of hypercitrullination of MBP in the pathogenesis of MS, researches reported that elevated serum citrulline levels in MS, and the structural similarity of Hcit with citrulline and immunogenic properties of Hcit, we aimed to contribute to the elucidation of the role of protein carbamylation in the pathogenesis of MS by inves-

Table 1. Basic clinical and demographic properties of patients with multiple sclerosis and healthy controls.

	RRMS group(n=44)	SPMS(n=36)	Control group(n=60)
Age(mean±SD, years)*	36.73 ± 6.87	39.34 ± 5.98	37.62 ± 5.52
Sex (n)			
Female	30	24	39
Male	14	12	21
Disease duration (median(min-max) year)#	2(1-20)	10(1-25)	
EDSS(mean ±SD)*	1.93 ± 1.39	5.20 ± 2.02	
Drugs(n%)			
Interferon	27.58%		
Glatiramer acetate	25.19%		
Teriflunomida	13.24%		
Fingolimod	25.95%	32.88%	
Ocrelizumab		68.20%	
No treatment	13.18%		

*: Parameters were expressed as mean ±SD, #: Parameters were expressed as median(min-max).

Table 2. Comparison of serum Hcit levels of participants.

Parameters	MS (n=80)	Control (n=60)	p
WBC (10^9/L) *	7.45 ± 2.70	7.35 ± 1.95	0.806
MPV (fL)*	8.99 ± 1.56	8.58 ± 0.83	0.040
LYM (10^9/L)*	1.65 ± 0.85	2.15 ± 0.63	<0.001
NEU (10^9/L)*	4.81 ± 2.60	4.04 ± 1.61	0.025
MONO (10^9/L) *	0.546 ± 0.20	0.511 ± 0.50	0.240
PLR#	166.29 (56.25-1326.67)	126.66 (48.92-376.25)	<0.001
NLR#	2.63 (0.79-27.71)	1.78 (0.78-6.82)	<0.001
MLR#	0.340 (0.03-2.50)	0.240 (0.06-0.70)	<0.001
RDW (%)*	15.08 ± 2.83	13.97 ± 1.51	0.003
HGB (g/L) *	13.76 ± 1.58	13.87 ± 1.46	0.664
PLT (10^9/L) *	283.75 ± 63.64	268.67 ± 70.40	0.160
ALT (U/L) *	24.21 ± 14.75	22.81 ± 18.0	0.595
AST (U/L) *	22.82 ± 9.34	21.81 ± 6.76	0.438
GLU (mg/dL) *	93.87 ± 25.24	101.96 ± 55.02	0.301
Urea (mg/dL) *	26.57 ± 8.52	26.12 ± 7.18	0.717
CREA (mmol/L) *	0.87 ± 0.15	0.85 ± 0.18	0.493
eGFR (ml dakika/1.73m2) *	114.79 ± 17.94	110.04 ± 20.48	0.122
TC (mg/dL) *	195.49 ± 32.74	185.02 ± 38.31	0.105
TG (mg/dL) *	120.80 ± 77.09	116.68 ± 62.34	0.720
LDL-C (mg/dL)*	104.56 ± 24.23	101.73 ± 33.66	0.553
HDL-C (mg/dL) *	54.03 ± 13.16	50.75 ± 11.96	0.210
Hcit (µmol / mol lizin)#	301.42 (105.40-875.80)	187.15 (53.29-450.50)	<0.001

*: Parameters were expressed as mean ±SD and compared with Student's t-test, #: Parameters were expressed as median(min-max) and compared with Mann-Whitney U test.

tigating serum Hcit levels in MS patients. Current study demonstrated that increased serum Hcit concentrations in patients with MS. Comparison of MS subgroups according to Hcit levels showed that serum Hcit concentrations were higher in patients with SPMS than in patients with RRMS. There was a positive correlation between serum Hcit levels with EDSS and disease duration. Therefore, it may be considered that Hcit residues may occur as a result of carbamylation of lysine residues in MBP in patients with MS, and that increased secretion of Hcit from the brain may be a potential marker in the pathogenesis and progression of MS. However, further studies are needed to prove the exact mechanism.

MPO is a part of the heme peroxidase superfamily that is predominantly expressed in neutrophils and monocytes [30]. There was a link elevated MPO levels, increased inflammation and oxidative stress, and increased MPO levels and activity have been reported in various autoimmune diseases, including the joints of patients with RA and the central nervous system of MS [31, 32]. In addition, there are studies showing that MPO activity is increased in patients with MS and that there is a correlation between

Table 3. Comparison of RRMS, SPMS and control groups.

Parameters	RRMS (n=44)	SPMS (n=36)	Control (n=60)	p		
				a	b	c
WBC (10^9/L)*	7.21 ± 2.02	7.81 ± 3.50	7.35 ± 1.95	0.765	0.969	0.868
MPV (fL)*	8.88 ± 1.23	9.19 ± 1.99	8.58 ± 0.83	0.834	0.376	0.315
LYM (10^9/L)*	1.71 ± 0.93	1.55 ± 0.72	2.15 ± 0.63	0.766	0.016	<0.001
NEU (10^9/L)*	4.28 ± 1.60	5.62 ± 3.49	4.04 ± 1.61	0.141	0.801	0.057
MONO (10^9/L) *	0.511 ± 0.17	0.597 ± 0.23	0.511± 0.17	0.045	0.985	0.028
PLR#	175.85 (56.25-1326.67)	157.16 (84.78-572.0)	126.66 (48.92-376.25)	0.556	<0.001	0.003
NLR#	2.36 (0.83-22.0)	3.37 (0.79-27.71)	1.78 (0.78-6.82)	0.138	0.001	<0.001
MLR#	0.28 (0.06-2.50)	0.37 (0.03-2.0)	0.24 (0.06-0.70)	0.207	0.011	<0.001
RDW (%)*	15.46 ± 3.16	14.43± 2.05	13.97 ±1.51	0.271	0.011	<0.001
HGB (g/L)*	13.79 ± 1.38	13.72 ± 1.86	13.87 ± 1.46	0.849	0.779	0.651
PLT (10^9/L) *	303.10 ± 58.15	254.12 ± 60.97	268.67 ± 70.40	0.001	0.004	0.289
ALT (U/L)*	27.52 ± 15.15	19.47 ± 14.57	22.81 ± 15.37	0.104	0.369	0.332
AST (U/L)*	24.45 ± 9.06	20.46 ± 9.38	21.81± 6.76	0.099	0.234	0.847
GLU (mg/dL)*	93.50 ± 29.20	94.50 ± 17.53	101.96 ± 55.02	0.935	0.353	0.493
Urea (mg/dL)*	24.91 ± 6.53	28.97 ± 10.41	26.12 ± 7.18	0.160	0.707	0.414
CREA (mmol/L)*	0.88 ± 0.13	0.84 ± 0.19	0.85 ± 0.18	0.358	0.296	0.912
eGFR (ml dakika/1.73m2) *	112.35 ± 19.34	118.58 ± 15.04	110.04 ± 20.48	0.484	0.892	0.112
TC (mg/dL) *	195.46 ± 29.80	195.52 ± 36.89	185.02 ± 38.31	0.996	0.182	0.224
TG (mg/dL)*	117.39 ± 44.51	129.0 ± 51.54	116.68 ± 62.34	0.532	0.956	0.481
LDL-C (mg/dL)*	103.95 ± 20.28	105.57 ± 30.01	101.73 ± 33.66	0.992	0.955	0.923
HDL-C (mg/dL)*	56.52 ± 14.45	50.08 ± 10.15	50.75 ± 11.96	0.157	0.067	0.861
Hcit (µmol / mol lizin)#	251.66 (105.40-875.80)	358.0 (150.0-678.25)	187.15 (53.29-450.50)	0.010	0.001	<0.001

a: Comparison of RRMS and SPMS; b: Comparison of RRMS and control; c: Comparison of SPMS and control.

*: Parameters were expressed as mean ±SD and compared with One-way ANOVA analysis (post-hoc analysis with LSD or Tamhane's T2 tests, they were evaluated with Bonferroni correction and the level of significance was taken as p<0.017), #: Parameters were expressed as median(min-max) and compared with Kruskal – Wallis test (post-hoc analysis Mann-Whitney U, they were evaluated with Bonferroni correction and the level of significance was taken as p<0.017).

disease progression and MPO activity [32-35]. Therefore, increased circulating Hcit levels in patients with MS may also be associated with increased MPO activity in these patients.

Cyanate intervention decreased endothelial NO synthesis in human endothelial cells, and it increases tissue factor and plasminogen activator inhibitor-1 expression. In mice, cyanate administration has been shown to promote protein carbamylation, similar to uremic individuals, and reduce arterial vasorelaxation of aortic rings in response to acetylcholine. It was demonstrated that total endothelial nitric oxide synthase activity and nitric oxide synthesis were significantly decreased in aortic tissue of cyanate-treated mice. Therefore, it is thought that cyanate causes endothelial dysfunction. Due to the reactive nature of cyanate, its levels are usually evaluates by analyzing serum Hcit concentrations, and serum Hcit levels have recently been shown to be associated with cardiovascular disease [36]. However, it has been shown that endothelial function is significantly impaired in patients with MS and that impaired vascular function may promote the reduction in MS-related local blood flow by triggering the formation of atherosclerotic lesions. Therefore, increased Hcit and cyanate levels in patients with MS may indicate impaired endothelial dysfunction and decreased blood flow to the brain in these patients. This is the first study to investigate serum Hcit levels in patients with MS. Our study expressed that Hcit levels were significantly increased in patients with MS and that there was a signifcant correlation between disease activity and progression with Hcit levels. However, further studies are needed to elucidate the exact mechanism between Hcit and MS pathogenesis.

Conclusion

Elevated serum Hcit levels were determined in patients with MS. There was a correlation between serum Hcit levels and disease activity and duration of disease and inflammation markers. Therefore, our results show that Hcit may be a useful marker in the diagnosis, activation and progression of MS, and further studies are needed to elucidate the mechanisms leading to elevated serum Hcit levels in patients with MS.

Limitations

There are some limitations to our study. the number of participants can be increased. In addition, the inability to obtain cerebrospinal fluid samples of the participants and the lack of measurement of nitric oxide levels, MPO activity and histopathological data showing the degree of myelinization were the limitations of the study.

Competing interests

The authors declare that they have no competing interest.

Financial disclosure

There are no financial supports.

Ethical approval

This study was approved by Selcuk University Faculty of Medicine Ethics Committee local ethics committee (Number: 2021/514, Date: 07.12.2021) and this study was conducted in accordance with the Declaration of Helsinki.

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