

Serum vitamin D, vitamin D binding protein levels and leukocyte vitamin D receptor gene expression in patients with ischaemic stroke

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Abstract

Objective: To investigate the possible contributions of serum 25-hydroxyvitamin D and vitamin D binding protein levels along with leukocyte vitamin D receptor gene expression in patients with ischaemic stroke.

Methods: The case-control study was conducted from December, 2016, to February, 2017, at the Department of Emergency Medicine, Erciyes University, Turkey, and comprised acute ischaemic stroke patients and matching healthy volunteers as controls. Severity of ischaemic stroke was assessed at admission using the National Institutes of Health Stroke Scale score. Gene expression of vitamin D receptor in leukocyte was assessed by real time-polymerase chain reaction.

Results: Of the 90 subjects, 51 (56.6%) were cases with a mean age of 65.2 ± 14.3 years, and 39 (43.3%) were controls with a mean age of 61.1 ± 16.7 years. There was no difference between the groups with respect to vitamin D deficiency, serum vitamin D binding protein levels and leukocyte vitamin D receptor gene expressions ($p > 0.05$). A negative correlation was found between 25-hydroxyvitamin D levels and the severity of ischaemic stroke ($p = 0.0342$).

Conclusion: There was a correlation between serum 25-hydroxyvitamin D levels and severity of ischaemic stroke as assessed by the National Institutes of Health Stroke Scale.

Keywords: Ischemic stroke, Vitamin D, Vitamin D binding protein, Gene expression, Correlation.

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Introduction

Stroke remains one of the leading global causes of death and long-term disability. Approximately 80% of all stroke patients are regarded as having ischaemic stroke (IS).¹ The great majority of IS cases represent a multi-factorial complex disease caused by a combination of genetic and environmental risk factors.² Modifiable and non-modifiable risk factors for IS include gender, age, race/ethnicity, heredity, hypertension (HTN), atrial fibrillation (AF), diabetes mellitus (DM), hypercholesterolaemia, cigarette smoking, and alcohol abuse.³ The presence of impaired consciousness or

disorientation at discharge is associated with markedly worse outcomes after IS.⁴ Moreover, early neurological deterioration is associated with higher mortality in IS patients.⁵

Vitamin D is essential for the human body to maintain a balance between calcium and phosphorus. There are several studies that investigated the relationship between IS and vitamin D. Large population-based studies and meta-analysis showed that gradual diminishing plasma 25-hydroxyvitamin D [25(OH) D] concentrations were associated with gradual increasing risk of IS.⁶ However, the contribution of vitamin D binding protein (VDBP) levels and leukocyte vitamin D receptor (VDR) gene expression on IS development has not been evaluated. The current study was planned to elucidate the possible association between serum 25(OH)D, VDBP levels and acute IS, and to assess the contribution of leukocyte VDR gene expressions in this regard.

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Patients and Methods

The case-control study was conducted from December, 2016, to February, 2017, at the Department of Emergency Medicine, Erciyes University, Turkey, and comprised acute ischaemic stroke patients as the cases and matching healthy volunteers as the controls. After approval from the institutional ethics committee, the sample size was calculated using GPower 3.1.9.2⁷ based on difference between two independent means, and good enough to detect a significant difference between the groups at 80% power level, $d=0.6$ effect size, and type I error of 0.05.

Those included using random sampling on the basis of a computer-generated randomisation scheme, were patients with acute IS and healthy volunteers matched by age and gender. Diagnosis of patients with IS was based on clinical findings, and was confirmed radiologically by using computed tomography (CT) and magnetic resonance imaging (MRI) scans of the brain. Those with previous history of cerebral haemorrhage, cerebrovascular event, transient ischaemic attack (TIA) or haemorrhagic infarct, brain trauma, cardioembolism, an inflammatory or infectious disease, tumour, haematological disorder, autoimmune disorder, hepatic or renal disease, hyper or hypothyroid disorders or use of immune-suppressant, anti-coagulant, anti-inflammatory drugs or vitamin and/or calcium supplements in the preceding two months were excluded, and so were those not willing to participate. The controls were chosen from among those visiting our hospital for annual health check-ups and were free from IS, cerebrovascular disease, neurological abnormalities, autoimmune disorders, and immunological disease.

After taking informed consent from the participants, demographic information and clinical history of dyslipidaemia, DM, coronary heart disease, HTN, obesity and IS were collected using a structured questionnaire. The National Institute of Health Stroke Scale (NIHSS) score⁸ was used to determine IS severity in patients at the time of presentation. The scale scans the consciousness, view, visual field, facial paralysis, weakness of arm and leg, ataxia, sense, tongue, dysarthria and all neurological findings, including neglect. The total score ranges from 0 to 42 points, with higher scores suggesting greater neurological impairment.⁸ Blood samples were taken by venipuncture after an overnight fast, and protected from sunlight. After centrifugation, serum

samples were immediately stored at -80°C until further use. Standard laboratory methods were used for biochemical measurements. Serum 25(OH)D was measured using a competitive chemiluminescent immunoassay on a Beckman Coulter Access 2 Immunoassay Analyser (California, USA), with intra- and inter-assay coefficients of variation of $<10\%$. This assay has 100% cross-reactivity with both metabolites of vitamin D namely, 25(OH)D₂ and 25(OH)D₃, and so, measures total serum 25(OH)D contents. The 25(OH)D levels were determined to identify the vitamin D status. The serum level of 30 ng/ml 25(OH)D was regarded as a threshold value according to the Endocrine Society criteria.⁹ Vitamin D levels 20-30 ng/ml were accepted as vitamin D insufficiency. Subjects whose vitamin D levels <20 ng/ml were considered vitamin D deficient.⁹ Serum levels of VDBP were determined using an enzyme-linked immunosorbent assay (ELISA) by using commercial kits. The published normal reference range for VDBP concentrations is 300-600 $\mu\text{g/ml}$, or 30-60 mg/dl.¹⁰

Venous blood samples (5 ml) were collected into tubes with ethylenediaminetetraacetic acid (EDTA). Leukocytes were isolated by the osmotic lysis method and the resulting cell pellets were stored at -80°C until ribonucleic acid (RNA) extraction. Genomic RNA was extracted with TRIzol reagent (Invitrogen, USA) from blood samples, according to the protocol of the manufacturer. Each RNA sample was eluted with ribonuclease (RNase)-free water. After isolation, the amount and the quality of total RNA were determined using a spectrophotometer (ND-1000, NanoDrop Technologies, USA) at 260/280 nm, and stored at -80°C . Complementary deoxyribonucleic acid (cDNA) was generated using Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics, Germany), according to the instructions of the manufacturer. Pre-amplification was performed using cDNA Pre-AMP Master Kit (Roche, Germany). Quantitative gene expression of VDR in leukocyte was detected by real-time polymerase chain reaction (PCR). As a template, the 5 ml of the synthesised cDNA was used for real-time PCR. The template was added to the reaction mixture (LightCycler[®] 480 Probes Master Mix, Roche, Germany) for real-time PCR. Quantitative gene expression profile was obtained by using LightCycler[®] 480 II (Roche Diagnostics, Mannheim, Germany). The experiment comprised the steps of the reaction mixture incubated for 2 min at 50°C for the

initial step, for 10 min at 95°C for deactivation and subsequently 45 cycles of 10 sec each at 95°C, 30 sec at 60°C, 1 sec at 72°C for denaturation and 30 sec at 40°C for cooling. PCR analysis and calculation of the quantification threshold cycle (Ct) values for relative quantification were carried out with the LightCycler 480 software version 1.5. Thus, Ct values were found for each sample. The experiment was performed in duplicates for both target gene and beta-actin (ACTB) as a housekeeping gene. Results of the target gene expression levels were expressed as $2^{\Delta\Delta Ct}$ in which $\Delta Ct = (Ct_{VDR} - Ct_{ACTB})$.

Results were presented as the mean $\pm$ standard deviation (SD) or frequencies and percentages. Chi-square test or Fisher's exact test was used for calculation of the significance of differences in categorical data. The groups were compared with each other for quantitative data by unpaired student's t test. Gene expression data was analysed using student's t test. Pearson's test was used to carry out correlation analysis. Statistical analysis was carried out using Graph-Pad Instat version 3.05. Results with two-tailed $p < 0.05$ were regarded as statistically significant.

Table-1: Demographic, clinic, and laboratory characteristics of the study participants.

	Controls (n=39)	Patients (n=51)	p-value
Mean age (years)*	61.1 $\pm$ 16.7	65.2 $\pm$ 14.3	0.2125
Gender			
Female (n, %)	15 (38.5)	19 (37.3)	
Male (n, %)	24 (61.5)	32 (62.7)	0.9069
BMI (kg/m ²)*	24.5 $\pm$ 7.1	22.7 $\pm$ 3.6	0.1205
Blood Pressure			
Systolic BP (mm Hg)*	121.6 $\pm$ 15.2	118.9 $\pm$ 9.3	0.3011
Diastolic BP (mm Hg)*	82.9 $\pm$ 12.1	79.4 $\pm$ 9.8	0.1331
Comorbidity			
Diabetes mellitus (n, %)	0 (0.0)	1 (2.0)	1.0000
Biochemical data			
Fasting plasma glucose (mg/dl)*	88.5 $\pm$ 13.6	84.7 $\pm$ 9.1	0.1164
HbA1c (%)*	5.9 $\pm$ 1.4	5.3 $\pm$ 1.9	0.1011
Total cholesterol (mg/dl)*	141.5 $\pm$ 45.7	150.6 $\pm$ 19.1	0.2023
Vitamin D (ng/ml)*	14.6 $\pm$ 9.5	13.5 $\pm$ 6.9	0.9614
VDBP (μ g/ml)*	306.8 $\pm$ 226.2	389.6 $\pm$ 502.6	0.9762
Score			
NIHSS score, Median (range)		3 (0-18)	
Electrocardiogram			
Atrial fibrillation (n, %)	0 (0.0)	10 (19.6)	
Normal sinus rhythm (n, %)	39 (100.0)	41 (80.4)	0.0042

*Data are mean $\pm$ SD. BMI, body mass index; BP, blood pressure; NIHSS, National Institutes of Health Stroke Scale; VDBP, Vitamin D binding protein; BMI: Body mass index; HbA1c: Glycated haemoglobin.

Results

Of the 90 subjects, 51 (56.6%) were cases with a mean age of 65.2 $\pm$ 14.3 years, and 39 (43.3%) were controls with a mean age of 61.1 $\pm$ 16.7 years. There was no difference between the groups with respect to gender, age, BMI, blood pressure (BP) vitamin D deficiency, serum VDBP levels and leukocyte VDR gene expressions ($p > 0.05$), but the patients group had a high incidence of AF (Table 1).

Vitamin D status in the control group was similar to the patients group ($p = 0.4610$).

NIHSS scores suggested 42 (82%) patients had mild to moderate impairment (score: 0-6), while 9 (18%) had moderate to severe deficits (score: 7-18). Serum 25(OH)D levels were negatively correlated with NIHSS score (Table 2), diminishing with increasing severity of stroke. No marked changes in leukocyte VDR gene expression were detected in patients with IS when compared to the controls ($p > 0.05$).

Table-2: Correlations between the Vitamin D, VDBP, VDR expression, and NIHSS score in patients with ischemic stroke.

Parameters	Correlation coefficient (r)	Coefficient of determination (r ²)	p-value
Vitamin D $\leftrightarrow$ VDBP	0.0916	0.0084	0.5541
Vitamin D $\leftrightarrow$ VDR expression	0.1177	0.0139	0.4815
VDBP $\leftrightarrow$ VDR expression	-0.0318	0.0010	0.8414
NIHSS $\leftrightarrow$ Vitamin D	-0.3201	0.1025	0.0342
NIHSS $\leftrightarrow$ VDBP	-0.0616	0.0038	0.6775
NIHSS $\leftrightarrow$ VDR expression	0.0194	0.0004	0.8995

NIHSS, National Institutes of Health Stroke Scale; VDBP, Vitamin D binding protein; VDR: vitamin D receptor

Discussion

We examined, for the first time in this study, the involvement of VDBP levels and leukocytes VDR gene expressions in IS development. There were no marked changes in VDBP levels and leukocytes VDR gene expressions in IS patients. No significant difference of serum 25(OH)D levels between the patients and their controls was observed. This finding is in line with the results of a previous study.¹¹ However, we observed that serum 25(OH)D levels were inversely correlated with severity of stroke as defined by the NIHSS score in the present study. Wang et al.¹² also showed that there is a negative relationship between the level of 25(OH)D and the infarct volume and the admission neurological deficit in patients with acute IS. Additionally, a recent study demonstrated that serum 25(OH)D levels were negatively associated with the risk of stroke recurrence and

mortality.¹³ Several studies have associated diminished 25(OH)D concentrations with increased risk of IS.^{6,14} Recent meta-analysis also showed that lower vitamin D status is associated with an increased risk of IS.¹⁵ It was reported that individuals with severe 25(OH)D deficiency had higher risk of IS compared to individuals with sufficient 25(OH)D level during 21 years of follow-up in a prospective population-based cohort study.⁶ We observed that the majority of patients and the controls had severe vitamin D deficiency. Our data supports the previously published findings, showing that vitamin D deficiency has high prevalence (75.54%) in the elderly and adult Turkish population.¹⁶ Collectively, serum 25(OH)D levels are considered independent predictors of functional outcome in acute IS patients.

Although VDR is accepted as one of potential candidates for cardiovascular diseases,¹⁷ its role in the IS pathogenesis remains to be identified. Our study found no change in leukocyte VDR gene expression in IS. Carbone et al.¹⁸ demonstrated that low intra-plaque VDR expression, but not circulating vitamin D level, predicts major adverse cardiovascular events in patients with carotid stenosis. VDR is a nuclear receptor, and it is an essential component for the effects of vitamin D. Following activation, VDR interact with vitamin D-responsive element, regulates target genes, and thereby modulates their transcriptional output.¹⁹ Vasoprotective effects of vitamin D and VDR activation were reported, including slowing down of atherosclerosis, suppression of the renin-angiotensin-aldosterone system, and promotion of endothelial cell function.²⁰

One key determinant of 25(OH)D levels is VDBP, a group-specific component of serum globulin.²¹ VDBP is the principal protein carrier for serum vitamin D, and acts as a reservoir for vitamin D metabolites. About 85-90% of 25(OH)D is transported from the liver to target organs bound to VDBP.²² In the present study, we showed that serum VDBP levels did not contribute to IS development.

The limitations of the current study were its small sample size and the non-use of vitamin D therapy. We detected basal serum vitamin D levels, and the deficiency was not corrected with vitamin D administration. Therefore, larger, prospective, randomised controlled clinical trials are required to assess the role of vitamin D in acute IS.

Conclusion

Serum 25(OH)D levels did not change markedly in IS.

This finding was related to low levels of serum 25(OH)D in the control subjects. There was an inverse correlation of serum 25(OH)D levels with severity of IS as assessed by the NIHSS. Besides, low vitamin D level is not an established risk factor for IS.

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