Journal of Molecular Neuroscience
https://doi.org/10.1007/s12031-018-1059-5

Expression Analysis of Vitamin D Signaling Pathway Genes in Epileptic Patients

Mehrdokht Mazdeh[1,2] · Soudeh Ghafouri-Fard[3] · Mahsa Hatami[3] · Mohammad Mahdi Eftekharian[1] · Maziar Ganji[3] · Arezou Sayad[3] · Shahram Arsang-Jang[4] · Mohammad Taheri[3,5] · Mir Davood Omrani[3,5]

Received: 10 February 2018 / Accepted: 7 March 2018

Abstract

Vitamin D deficiency has been detected in epileptic patients. Vitamin D participates in neuroprotection, brain cell proliferation, and differentiation. Consequently, vitamin D supplementation has been suggested as an alternative treatment in epileptic patients. We aimed at assessment of vitamin D signaling pathway in epileptic patients. In the present study, we evaluated vitamin D serum concentration as well as expression of vitamin D receptor (*VDR*) gene and genes encoding for vitamin D activating enzyme 1-alpha-hydroxylase (CYP27B1) and deactivating enzyme 24-hyroxylase (CYP24A1) in epileptic patients compared with healthy individuals. We found significant lower levels of vitamin D in epileptic patients compared with healthy subjects. Expression analyses showed significant downregulation of *VDR* expression in peripheral blood of epileptic patients compared with healthy subjects (relative expression (REx) = 0.16, $P < 0.001$). However, there was no significant difference in *CYP24A1* expression between epileptic patients and normal subjects. *CYP27B1* expression analysis showed significant upregulation in male patients aged between 30 and 40 (REx = 5.43, $P = 0.013$). After using two-way ANCOVA for adjusting the effects of sex and age, there was a statistically significant difference in the *VDR* expression values between patient and control groups ($P < 0.001$). Spearman's correlation analysis showed no significant correlation between genes expression levels and patients' age or vitamin D serum concentrations. However, we found significant correlations between *VDR* expression levels and *CYP24A1*/ *CYP27B1* expression levels in epileptic patients ($r = 0.435$ and $P < 0.001$; $r = 0.26$ and $P = 0.02$ respectively). There was also a significant correlation between the expression levels of *CYP24A1* and *CYP27B1* ($r = 0.349$ and $P = 0.001$). Our study shows a possible role for VDR in the pathogenesis of epilepsy.

Keywords Vitamin D · VDR · CYP27B1 · CYP24A1 · Epilepsy

Introduction

Epilepsy as a brain disorder resulting in repetitive seizures is regarded as an important health problem. Epileptic patients suffer from noticeable and consistent health and socioeconomic incongruity in spite of novel treatment approaches and social awareness attempts. Statistics show the presence of active epilepsy in 1.2% of the US population (Zack and Kobau 2017). A significant proportion of epileptic patients have drug-resistant epilepsy which results in mental and mood disturbances, traumas, and elevated risk of death (Pendo and DeGiorgio 2016). Because of the toxicity of the available antiepileptic drugs (AEDs), researchers have focused on identification of alternative treatments for epilepsy (Pendo and DeGiorgio 2016). Evidences from ecological, interventional, and animal studies have demonstrated association between vitamin D deficiency and epilepsy (Hollo et al. 2014). Consequently,

Mehrdokht Mazdeh and Soudeh Ghafouri-Fard contributed equally to this work.

✉ Mohammad Taheri
mohammad_823@yahoo.com

✉ Mir Davood Omrani
davood_omrani@yahoo.co.uk

[1] Neurophysiology Research Center, Hamadan University of Medical Sciences, Hamadan, Iran

[2] Department of Neurology, Hamadan University of Medical Sciences, Hamadan, Iran

[3] Department of Medical Genetics, Shahid Beheshti University of Medical Sciences, Tehran, Iran

[4] Clinical Research Development Center (CRDU), Qom University of Medical Sciences, Qom, Iran

[5] Urogenital Stem Cell Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Published online: 16 March 2018

Table 1 Nucleotide sequences of primers and probes used for amplification of each segment

Gene name	Product length	Primer and probe sequences
HPRT1	F:AGCCTAAGATGAGAGTTC R: CACAGAACTAGAACATTGATA	88
	FAM-CATCTGGAGTCCTATTGACATCGC-TAMRA	
VDR	F: TGGCTTTCACTTCAATGCTATGA R: CGTCGGTTGTCCTTGGTGAT	126
	FAM-ACTTCCGGCCTCCAGTTCGTATGGAC-TAMRA	
CYP27B1	F: CCCAGATCCTAACACATTTTGAGG R:AAAGGGTGATGATGACAGTCTCTTTC	152
	FAM- ACCCAAGACCCGGACTGTCCTGGT-TAMRA	
CYP24A1	F: TATCGCGACTACCGCAAAGA R: CGGCCAAGACCTCATTGATT	145
	FAM-TCCGGACCCGCTGCCAGTCTT-TAMRA	

vitamin D supplementation has been suggested as a putative alternative epilepsy treatment (Pendo and DeGiorgio 2016). Further evidences supporting the role of vitamin D as in the treatment of epilepsy have emerged from studies demonstrating its participation in neuroprotection, brain cell proliferation, and differentiation as well as detection of vitamin D-specific receptors and enzymes in neurons and glial cells of the central/peripheral nervous system (Pendo and DeGiorgio 2016). The hormonally active form of vitamin D is 1,25-dihydroxy-vitamin D [1,25$(OH)_2$D] which exert its functional role via binding to the nuclear vitamin D receptor (VDR). The metabolic pathway of vitamin D includes two other important proteins coded by *CYP27B1* and *CYP24A1* genes. The protein coded by *CYP27B1* transforms 25-hydroxy-vitamin D [25(OH)D] into the active vitamin D metabolite 1,25$(OH)_2$D which is subsequently inactivated by the protein coded by *CYP24A1* (Holick et al. 2007).

In the present study, we aimed at assessment of vitamin D serum concentration as well as expression analysis of *VDR* gene and genes encoding for vitamin D activating enzyme 1-alpha-hydroxylase (CYP27B1) and deactivating enzyme 24-hyroxylase (CYP24A1) in epileptic patients compared with healthy individuals.

Methods

Participants

The current study is a case-control study of 40 patients affected with generalized epilepsy involving both cerebral hemispheres from onset of disorder (19 females and 21 males, age mean: 36.66 ± 2.8 years) and 40 age-/gender-matched healthy controls. All patients were outpatients of the department of Neurology, Hamadan University of Medical Sciences. All of them were under treatment with Depakene (valproic acid) as the only AED and did not have any seizure attack in the 6 month period prior to sampling. Epilepsy was diagnosed by neurologists according to patients' report of seizure occurrence as well as findings of electroencephalogram (EEG) and brain magnetic resonance imaging (MRI). Patients had no other medical problem such as history of head trauma. Patients with poor compliance with AED, inconstant record of seizure rate, history of pseudo seizures, alcohol or drug abuse, any serious medical problem such as any kind of malignancy, hepatic, or renal failure were excluded from the study. The local ethical committee approved the study. Informed consent forms were provided by all participants.

Table 2 Demographic and clinical data of patients and controls

Variables	Patients	Controls
Female/male [no. (%)]	19 (47.5%)/21(52.5%)	19 (47.5%)/21(52.5%)
Age (mean ± SD, years)	36.66 ± 2.8	34.06 ± 1.9
Age range (years)	21–58	23–62
Age at onset (mean ± SD, years)	28 ± 8.6	–
Generalized epilepsy (no. %)	100 (100%)	–
Disease duration (mean ± SD, years)	8.18 ± 4.1	–

Table 3 *VDR* relative expression in epileptic patients compared with healthy subjects

VDR expression		Patient numbers	Control numbers	Expression ratio	Standard deviation	*P* value	95% confidence intervals
Total		40	40	0.1674	0.725	< 0.001	[− 4.55 – 1.7]
Male		19	19	0.1131	1.069	0.001	[− 5.96 – 1.75]
Female		21	21	0.2394	1.05	0.017	[− 4.54 – 0.407]
< 30	Male	3	12	0.1028	4.16	0.075	[− 10.8 2.47]
	Female	5	10	0.6128	1.46	0.556	[− 3.9 1.89]
30–40	Male	11	3	0.3107	3.9	0.255	[− 6.21 2.42]
	Female	9	10	0.2015	1.78	0.088	[− 6.25 0.81]
> 40	Male	5	4	0.0137	2.52	0.003	[− 12.3− 2.43]
	Female	7	1	0.334	–	–	–

Assessment of Serum Vitamin D Level

Serum vitamin D levels were assessed by electrochemiluminescence method using commercial kits (Roche Elecsys 2010 Chemistry Analyzer, Roche, Germany). Based on serum vitamin D levels, study participants were classified into three groups of normal (vitamin D levels > 30 ng/ml), insufficient (vitamin D levels 20–30 ng/ ml), and deficient (vitamin D levels < 20 ng/ ml) and compared with each other. Subsequently, subjects of insufficient and deficient groups were combined and analysis was repeated between these subjects and those with normal vitamin D levels.

VDR, *CYP24A1*, and *CYP27B1* Expression Analysis

Five milliliters of peripheral blood samples were collected from the participants in EDTA tubes. GeneAll Hybrid-RTM blood RNA extraction kit (cat No.305-101) was used for total RNA extraction based on the manufacturer's protocol. The quality of extracted RNA samples was assessed by Eppendorf Biophotometer Plus (Thomas Scientific, Germany) and electrophoresis on 1% agarose gel. The cDNA first strand was synthesized using Hyper Script™ First Strand Synthesis Kit (Cat. No. 605-005) based on the manufacturer's instructions. Specific primers and probes for the *VDR*, *CYP24A1*, *CYP27B1*, and hypoxanthine phosphoribosyl transferase (*HPRT1*) were designed by allele ID 7 software program (Premier Biosoft, Palo Alto, USA). *HPRT1* gene was chosen as the reference gene for normalization of expression levels of mentioned genes. Real-time quantitative PCR was carried out in the Corbett Rotor Gene 6000 machine (Corbett Life Science) using the BiosystemsTaqMan®, Universal PCR Master Mix (PN: 4304449). The nucleotide sequences of primers and probes are summarized in Table 1.

Statistical Analysis

The expression levels of mentioned genes in epileptic patients were compared with normal subjects by using the independent *t* test. To test the significance of difference in mean values between patients and control groups, OpenBUGS 3.2.2 (OpenBUGS Foundation, London, UK) was used to fit two-sample Bayesian *t* test. A normal prior distribution was assumed for parameters with 20,000 iterations. Spearman's correlation coefficient was used to identify the level of correlation between the variables. Analysis of covariance (ANCOVA) was used to analyze the main and interaction effects of categorical variables on gene expression in order to control the effects of selected variables such as age and sex. The level of

Table 4 *CYP24A1* relative expression in epileptic patients compared with healthy subjects

CYP24A1 expression		Patient numbers	Control numbers	Expression ratio	Standard deviation	*P* value	95% confidence intervals
Total		40	40	1.4361	0.76	0.135	[− 0.906 2.11]
Male		19	19	0.9163	1.21	0.714	[− 2.62 2.18]
Female		21	21	2.1301	1.02	0.171	[− 0.65 3.38]
< 30	Male	3	12	0.8347	4.5	0.775	[− 7.09 7.45]
	Female	5	10	1.916	1.9	0.58	[− 2.71 4.87]
30–40	Male	11	3	4.6024	5.8	0.114	[− 3.86 9.61]-
	Female	9	10	1.29	1.65	0.756	[− 2.81 3.74]
> 40	Male	5	4	0.066	4.1	0.072	[− 12.9 3.09]
	Female	7	1	10.392	–	–	–

Table 5 *CYP27B1* relative expression in epileptic patients compared with healthy subjects

CYP27B1 expression		Patient numbers	Control numbers	Expression ratio	Standard deviation	*P* value	95% confidence intervals
Total		40	40	1.313	0.648	0.533	[− 0.88 1.66]
Male		19	19	1.2882	0.347	0.725	[− 1.67 2.36]
Female		21	21	1.3349	1.048	0.603	[− 0.53 2.61]
< 30	Male	3	12	1.1752	0.211	0.925	[− 7.09 7.45]
	Female	5	10	1.9452	2.76	0.448	[− 4.38 6.51]
30–40	Male	11	3	5.4304	3.69	0.013	[1.33 5.32]
	Female	9	10	0.8183	1.049	0.655	[− 2.52 1.64]
> 40	Male	5	4	0.2311	3.86	0.29	[− 10.27 4.81]
	Female	7	1	3.1264	–	–	–

significance was set at P values ≤ 0.05. The statistical analysis was implemented using SPSS version 18 (Chicago, IL, USA).

Results

Demographic and clinical data of study participants are summarized in Table 2. The mean values of serum vitamin D concentrations were compared between two study groups using independent t test which showed the significant higher levels of vitamin D in healthy subjects compared with epileptic patients (mean values of 25.76 and 31.15 for patients and controls respectively, $P = 0.003$). The average odds of enrolling in the control group for an individual is 1.09 times of the odds for case group with one unit increase in vitamin D level, after holding all other variables constant. Expression analyses showed significant downregulation of *VDR* expression in peripheral blood of epileptic patients compared with healthy subjects (relative expression (REx) = 0.16, $P < 0.001$). This downregulation was also significant in both male and female patients as well as in male patients aged > 40 compared with the corresponding control subjects. However, there was no significant difference in *CYP24A1* expression between epileptic patients and normal subjects. *CYP27B1* expression analysis showed significant upregulation in male patients aged between 30 and 40 (REx = 5.43, $P = 0.013$). The results of *VDR*, *CYP24A1*, and *CYP27B1* expression levels in epileptic patients compared with normal subjects are shown in Tables 3, 4, and 5 respectively. After using two-way ANCOVA for adjusting the effects of sex and age, there was a statistically significant difference in the *VDR* ΔΔCT values between patient and control groups ($P < 0.001$) but not for *CYP24A1* and *CYP27B1* ΔΔCT values. There was no interaction effect between disease status and sex or age which shows no age-/sex-related difference in gene expression in each study group. In addition, Spearman's correlation analysis showed no significant correlation between *VDR*, *CYP24A1*, or *CYP27B1* expression levels and patients' age (Figs. 1, 2, and 3 respectively). No significant correlation was found between *VDR*, *CYP24A1*, or *CYP27B1* expression levels and vitamin D serum concentrations (Figs. 4, 5, and 6 respectively). Moreover, we analyzed correlations between *VDR* expression levels and *CYP24A1*/*CYP27B1* expression levels in epileptic patients and found significant correlation in both cases ($r = 0.435$ and

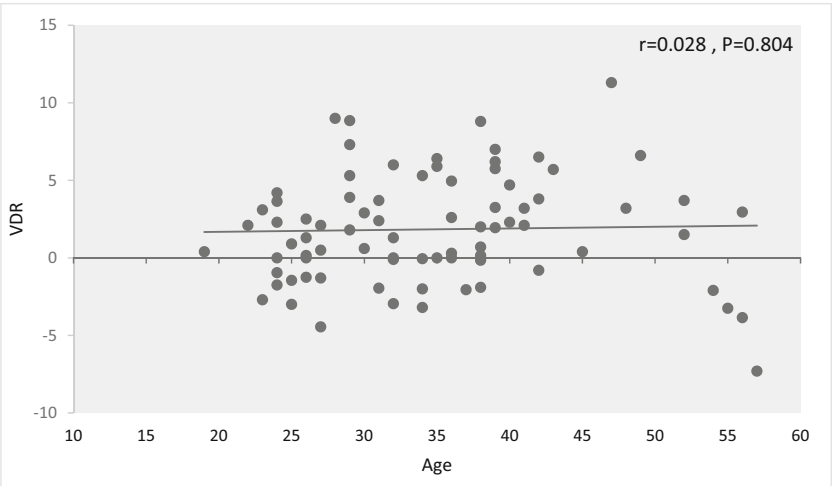

Fig. 1 Spearman's correlation analysis between *VDR* expression levels and patients' age

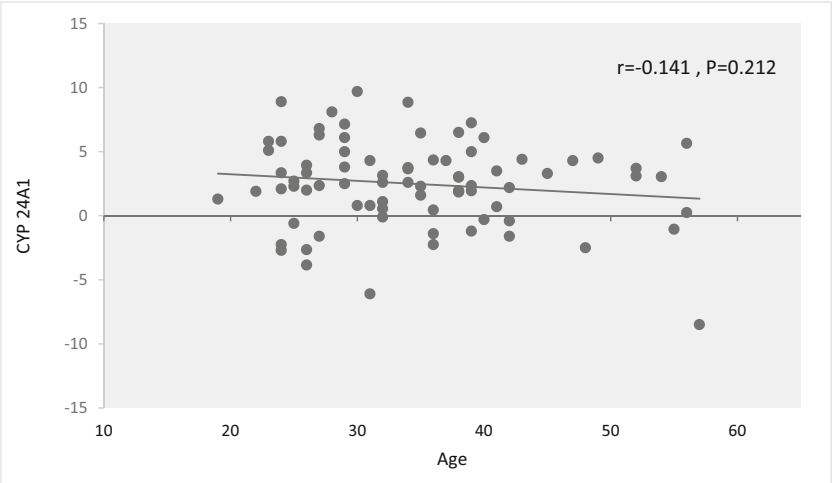

Fig. 2 Spearman's correlation analysis between *CYP24A1* expression levels and patients' age

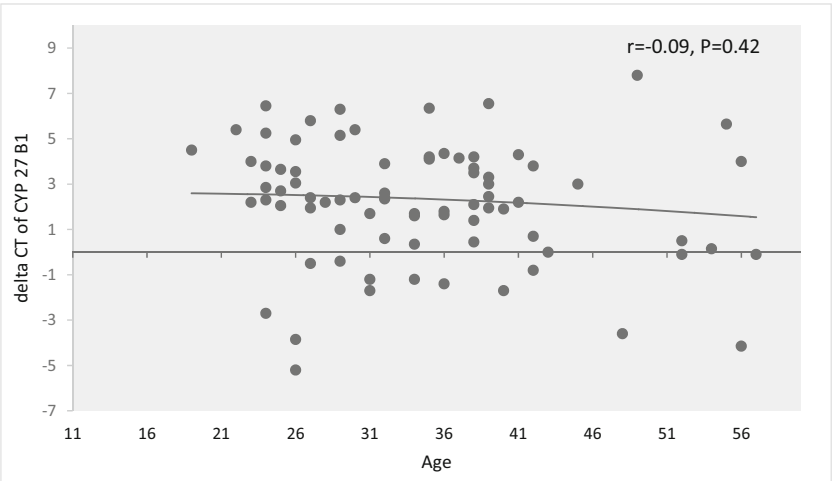

Fig. 3 Spearman's correlation analysis between *CYP27B1* expression levels and patients' age

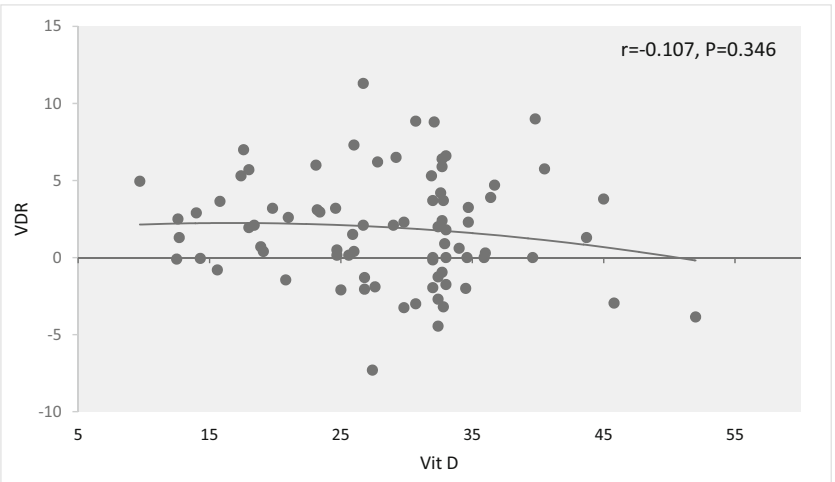

Fig. 4 Spearman's correlation analysis between *VDR* expression levels and vitamin D serum concentrations

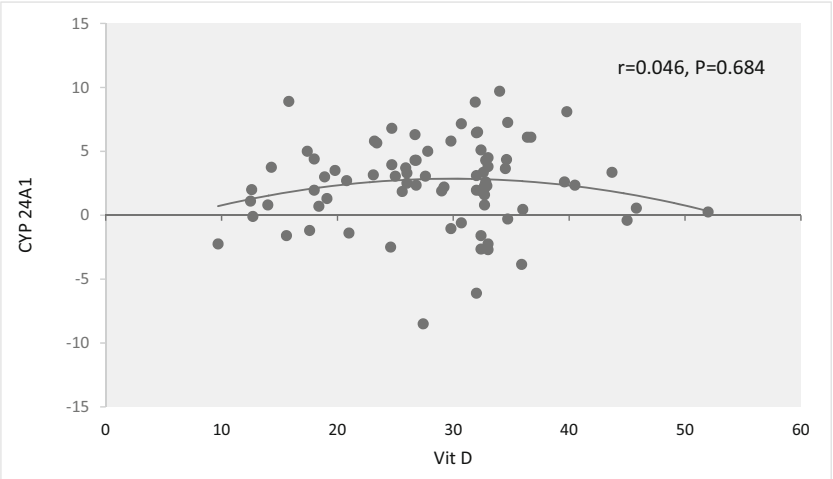

Fig. 5 Spearman's correlation analysis between *CYP24A1* expression levels and vitamin D serum concentrations

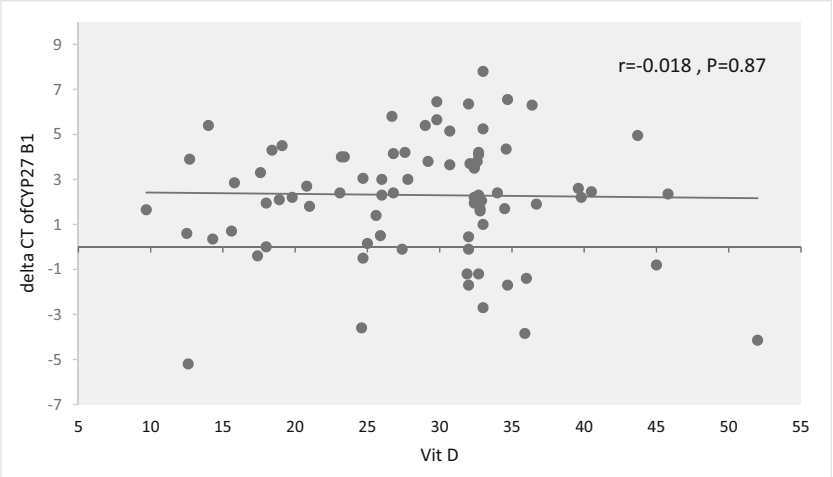

Fig. 6 Spearman's correlation analysis between *CYP27B1* expression levels and vitamin D serum concentrations

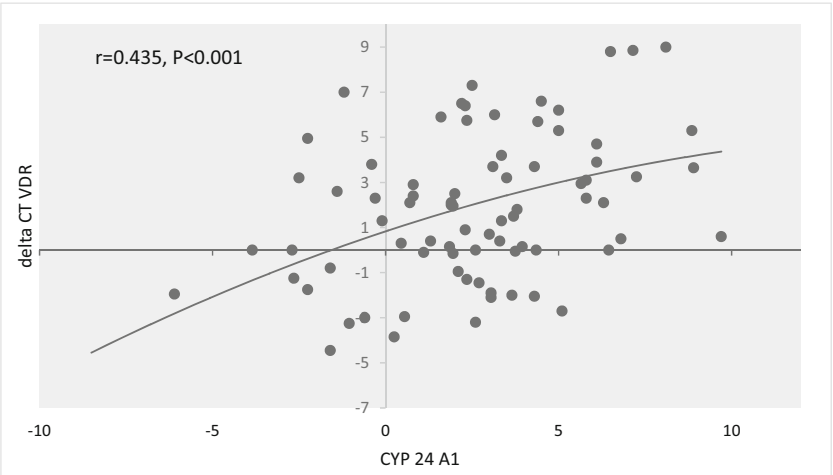

Fig. 7 Spearman's correlation between *VDR* and *CYP24A1* expression levels in patients

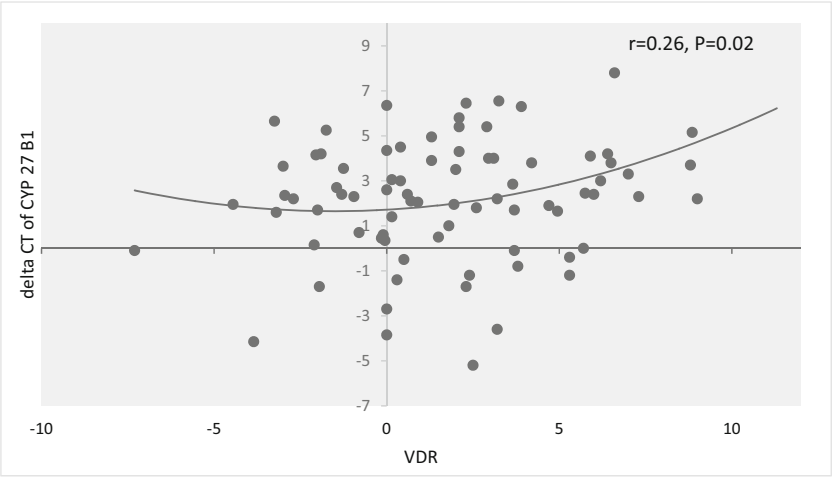

Fig. 8 Spearman's correlation between *VDR* and *CYP27B1* expression levels in patients

$P < 0.001$; $r = 0.26$ and $P = 0.02$ respectively) (Figs. 7 and 8 respectively). There was a significant correlation between the expression levels of *CYP24A1* and *CYP27B1* ($r = 0.349$ and $P = 0.001$) (Fig. 9).

Discussion

In the present study, we demonstrated lower levels of serum vitamin D as well as significant downregulation of *VDR* expression in peripheral blood of epileptic patients compared with healthy subjects. Such disturbed vitamin D processing by peripheral blood cells of epileptic patients may increase their susceptibility to a poor vitamin D condition. *VDR* polymorphisms have been shown to be associated with epilepsy previously (Jiang et al. 2015). VDR has been shown to be expressed in various blood cells, and the hormonal form of vitamin D, $1,25(OH)_2D$ has crucial role in the appropriate regulation of the immune system (Janik et al. 2017). Peripheral blood cells such as monocytes, dendritic cells, $CD4^+$ and $CD8^+$ T cells, and B cells express CYP27B1 and CYP24A1 in addition to VDR and participate in maintaining serum $1,25(OH)_2D$ levels. Most importantly, they provide most of $1,25(OH)_2D$ produced locally in the tissues (Smolders et al. 2011). Consequently, expression analysis of these genes in peripheral blood of epileptic patients might provide clues for understanding regulation of vitamin D signaling pathway both in the blood and in target tissues such as the brain. The application of VDR and CYP27B1/CYP24A1 has been suggested as a reasonable tool for assessment of vitamin D processing in immune cells as well (Smolders et al. 2011). If we accept the role of immune system dysregulation in the pathogenesis of epilepsy, the inflammatory process is expected to be initiated from peripheral blood cells leading to autoimmune mechanisms within the brain.

Vitamin D deficiency has been associated with both increased risk of autoimmune disorders and vulnerability to infections (Aranow 2011). On the other hand, autoimmunity has been proposed to participate in the pathogenesis of at least some kinds of human epilepsies (Cojocaru and Cojocaru

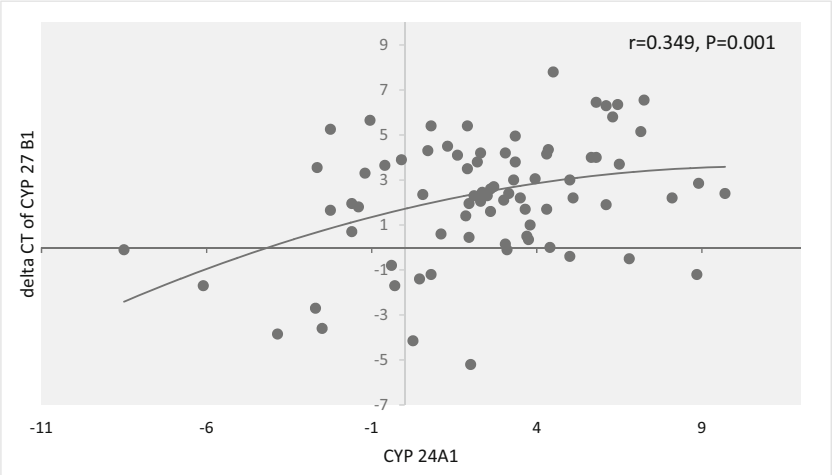

Fig. 9 Spearman's correlation between *CYP27B1* and *CYP24A1* expression levels in patients

2010). Considering the abundance of data regarding associations between vitamin D deficiency and epilepsy (Shellhaas et al. 2010), it is tempting to speculate that vitamin D deficiency might be involved in epilepsy pathogenesis via dysregulation of immune responses. However, there are some other proposed functions for vitamin D in nervous system including neuroprotection, brain cell proliferation, and differentiation inappropriate function of all might participate in the pathogenesis of epilepsy (Pendo and DeGiorgio 2016).

In addition, we found significant correlations between *VDR* expression levels and *CYP24A1*/*CYP27B1* expression levels as well as a significant correlation between the expression levels of *CYP24A1* and *CYP27B1* in epileptic patients. A positive correlation has been previously detected between CYP24A1 and CYP27B1 protein expression in thyroid tissues (Clinckspoor et al. 2012). Besides, in a previous study on peripheral blood cells in multiple sclerosis patients, the CYP27B1/CYP24A1 ratio demonstrated a trend towards a correlation. Within the mononuclear cells, positive correlations have been detected between VDR expression and CYP27B1 expression, VDR and the CYP27B1/CYP24A1 ratio, but not between VDR and CYP24A1 (Smolders et al. 2011). The same study demonstrated no effect of gender and age on expression of VDR, CYP27B1, and CYP24A1 in patients or healthy individuals (Smolders et al. 2011) which is in line with our results.

We assessed expression analysis in a homogenous population of epileptic patients all of them being responsive to Depakene (valproic acid). Valproic acid has been previously shown through in vitro studies to increase expression of both *VDR* and *CYP24* mRNA in the presence of physiological amounts of vitamin D (Vrzal et al. 2011). In the present study we, did not find significant difference in *CYP24A1* expression between patients and healthy subjects to propose possible effect of AED on expression of these genes. More importantly, we demonstrated significant lower expression of *VDR* in patients compared with controls which implies that downregulation of *VDR* in epileptic patients might have been even more prominent if we had excluded the effect of valproic acid on *VDR* expression. Consequently, we suggest that downregulation of VDR has a pathogenic role in epilepsy through attenuation of physiologic functions of vitamin D in neuroprotection or regulation of immune system. In addition, the impaired vitamin D signaling in epileptic patients as demonstrated by our study might indicate a need for higher vitamin D levels in epileptic patients compared with healthy subjects which should be provided through higher doses of supplements.

Funding Information The study was funded by Vice-chancellor for Research and Technology, Hamadan University of Medical Sciences (No. 9412187285).

References

Aranow C (2011) Vitamin D and the immune system. J Investig Med 59: 881–886

Clinckspoor I, Hauben E, Verlinden L, Van Den Bruel A, Vanwalleghem L, Poorten VV, Delaere P, Mathieu C, Verstuyf A, Decallonne B (2012) Altered expression of key players in vitamin d metabolism and signaling in malignant and benign thyroid tumors. J Histochem Cytochem 60:502–511

Cojocaru IM, Cojocaru M (2010) Reactions of the immune system in epilepsy. Maedica 5:201

Holick CN, Stanford JL, Kwon EM, Ostrander EA, Nejentsev S, Peters U (2007) Comprehensive association analysis of the vitamin D pathway genes, VDR, CYP27B1, and CYP24A1, in prostate cancer. Cancer Epidemiol Biomark Prev 16:1990–1999

Hollo A, Clemens Z, Lakatos P (2014) Epilepsy and vitamin D. Int J Neurosci 124:387–393

Janik S, Nowak U, Laszkiewicz A, Satyr A, Majkowski M, Marchwicka A, Sniezewski L, Berkowska K, Gabrys M, Cebrat M and Marcinkowska E (2017) Diverse regulation of vitamin D receptor gene expression by 1,25-Dihydroxyvitamin D and ATRA in murine and human blood cells at early stages of their differentiation. Int J Mol Sci 18. https://doi.org/10.3390/ijms18061323

Jiang P, Zhu WY, He X, Tang MM, Dang RL, Li HD, Xue Y, Zhang LH, Wu YQ, Cao LJ (2015) Association between vitamin D receptor gene polymorphisms with childhood temporal lobe epilepsy. Int J Environ Res Public Health 12:13913–13922

Pendo K, Degiorgio CM (2016) Vitamin D3 for the treatment of epilepsy: basic mechanisms, animal models, and clinical trials. Front Neurol 7:218

Shellhaas RA, Barks AK, Joshi SM (2010) Prevalence and risk factors for vitamin D insufficiency among children with epilepsy. Pediatr Neurol 42:422–426

Smolders J, Thewissen M, Theunissen R, Peelen E, Knippenberg S, Menheere P, Cohen Tervaert JW, Hupperts R, Damoiseaux J (2011) Vitamin D-related gene expression profiles in immune cells of patients with relapsing remitting multiple sclerosis. J Neuroimmunol 235:91–97

Vrzal R, Doricakova A, Novotna A, Bachleda P, Bitman M, Pavek P, Dvorak Z (2011) Valproic acid augments vitamin D receptor-mediated induction of CYP24 by vitamin D3: a possible cause of valproic acid-induced osteomalacia? Toxicol Lett 200:146–153

Zack MM, Kobau R (2017) National and state estimates of the numbers of adults and children with active epilepsy—United States, 2015. MMWR Morb Mortal Wkly Rep 66:821–825