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ORIGINAL ARTICLE

Expression analysis of vitamin D receptor-associated lncRNAs in epileptic patients

Mehrdokht Mazdeh[1] · Mehrdad Zamani[2] · Mohammad Mahdi Eftekharian[1] · Alireza Komaki[1] · Shahram Arsang-Jang[3] · Mohammad Taheri[4] · Soudeh Ghafouri-Fard[5]

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Abstract

Vitamin D has been vastly acknowledged as a neuroactive steroid controlling neurodevelopment. As it exerts its functions through activation of vitamin D receptor (VDR), several studies have assessed the role of VDR in brain function. More recently, a number of long non-coding RNAs (lncRNAs) have been recognized that alter expression of VDR. In the current study, we evaluated expression of four *VDR*-related lncRNAs (*LINC00511*, *LINC00346*, *SNHG6* and *SNHG16*) in peripheral blood of 40 epileptic patients and 39 healthy subjects using quantitative real time PCR method. The relative expression levels of *SNHG16* and *LINC00511* were higher in epileptic patients compared with healthy subjects. For *SNHG16*, the difference was only significant between male patients and male controls, while *LINC00511* had the opposite pattern. The results of Quantile regression model showed significant associations between *SNHG6* and *SNHG16* expressions and gender (*P* values of 0.027 and 0.009 respectively). Significant correlations were detected between expression levels of *SNHG6* and *SNHG16* (r = 0.32, *P* = 0.004), *SNHG6* and *LINC00346* (r = 0.37, *P* = 0.001), *SNHG16* and *LINC00346* (r = 0.30, *P* = 0.007) as well as *SNHG16* and *LINC00511* (r = 0.29, *P* = 0.009). Expression of *LINC00346* was inversely correlated with vitamin D levels only in male epileptic patients (r = −0.58, *P* = 0.011). Expression of *SNHG6* was correlated with vitamin D levels in male controls but no other subgroups (r = 0.51, *P* = 0.044). Based on the results of ROC curve analysis, *SNHG16* had the diagnostic power of 0.86 in male subjects. Taken together, the current study provides evidences for dys-regulation of VDR-related lncRNAs in epileptic patients. The clinical significance of these finding should be explored in future studies.

Keywords Epilepsy · VDR · LINC00511 · LINC00346 · SNHG6 · SNHG16 · lncRNA

Introduction

Epilepsy is a chronic neurological condition described by recurrent seizures caused by irrepressible neural discharges in the brain (Shimada et al. 2014). Although antiepileptic drugs (AEDs) are considered as chief treatment strategy for epilepsy, they are associated with diverse side effects such as allergies, mood disorders, memory loss and osteoporosis (Meier and Kraenzlin 2011; Reynolds 1975). Such complications have encouraged researchers to find alternative therapeutic option for epileptic patients. Vitamin D has been considered as an attractive target in this regard based on the results of animal studies and clinical trials (Pendo and Degiorgio 2016). Vitamin D has an underscored participation in several physiological functions in the brain tissue. For instance, vitamin D regulates proliferation and differentiation of neurons, calcium signaling, as well as neurotrophic and neuroprotective functions (Groves et al. 2014). The role

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✉ Mohammad Taheri
mohammad_823@yahoo.com

✉ Soudeh Ghafouri-Fard
s.ghafourifard@sbmu.ac.ir

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

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

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

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

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

Table 1 Nucleotide sequences of primers used in the study

Gene	Sequence 5′ → 3′	PCR product
B2M	F: AGATGAGTATGCCTGCCGTG R: GCGGCATCTTCAAACCTCCA	105 bp
SNHG16	F: GTCAGCCTCAGTTTCCAAAGC R: TAAAGACATGGCACTTTGGGTC	104 bp
SNHG6	F: AGGGAGGAAGAAGCGCGAA R: TCGCAGAGCCCAGCTACG	85 bp
LINC00511	F: TCCCACCAGGAAGTTTAGCAG R: GCCTCTCAAGAGGTGGTCC	87 bp
LINC00346	F: TGCCCTGGACATTCATGGAC R: CTGGACAAGCCCACTCTAGC	150 bp

of vitamin D signaling pathway in the epilepsy has been previously highlighted through the observed higher intensity of pentylenetetrazole-induced seizures in vitamin D receptor (*Vdr*) knock-out mice (Kalueff et al. 2006). More recently, certain polymorphisms within *VDR* gene has been associated with risk of epilepsy (Jiang et al. 2015) or the presence of vascular risk factors in epileptic patients (Phabphal and Geater 2013). We have recently performed an in silico data mining and identified a number of long non-coding RNAs (lncRNAs) that participate in the regulation of VDR signaling (Vahid et al. 2018). Moreover, we have verified their association with *VDR* in the context of breast cancer (Kholghi Oskooei et al. 2018). More recently, we reported down-regulation of *VDR* in peripheral blood of epileptic patients compared with healthy subjects (Mazdeh et al. 2018). In the current investigation, we analyzed expression of four *VDR*-related lncRNAs (*LINC00511*, *LINC00346*, *SNHG6* and *SNHG16*) in a population of Iranian epileptic patients and healthy subjects to explore their putative role in the pathogenesis of epilepsy.

Material and methods

Study participants

A total of 40 epileptic patients and 39 healthy subjects were enrolled in the current study. All patients had juvenile myoclonic epilepsy and have received valproic acid as monotherapy regimen for a period of 3–18 months. Patients did not have any seizure attack during the 6 month period before sampling. None of patients had febrile seizures. The diagnosis was performed according to electroencephalogram (EEG) and brain magnetic resonance imaging (MRI). Diffusion weighted (DW), T1, T2 and gradient eco images were analyzed. The local ethical committee approved the study. All participants signed informed consent forms. The presence of any neurological, psychiatric or systemic disorder was ruled out in persons attributed to control group. None of study participants were suffered from systemic or autoimmune disorders.

Expression assay

Five mL of whole venous blood was obtained through venipuncture from cases and controls in ethylene-diamine-tetra-acetic acid (EDTA) tubes. Total RNA was isolated from all specimens using Hybrid-RTM blood RNA extraction kit (GeneAll Biotechnology Co Ltd., South Koera). The quality and concentration of RNA samples were evaluated by gel electrophoresis and NanoDrop equipment (Thermo Scientific, MA, USA). Subsequently, RNA was converted to cDNA using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). Expression of *VDR*-related lncRNAs were quantified in the rotor gene 6000 corbett Real-Time PCR System by using SYBR® Premix Ex TaqTM (TaKaRa, Japan). *B2M* gene was used as normalizer. The nucleotides sequences of primers and PCR product length are demonstrated in Table 1.

Assessment of serum vitamin D level

Serum vitamin D levels were measured using commercial kit in Elecsys 2010 Chemistry Analyzer (Roche, Germany). According to serum vitamin D levels, study participants were categorized 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). Next, individuals with insufficient and deficient levels were combined and data was analyzed.

Table 2 Demographic and clinical data of patients and controls

Variables	Patients	Controls
Female/Male [no. (%)]	25 (62.5%) / 15(50%)	22(55%) / 18 (45%)
Age (mean ± SD, Y)	36.66 ± 2.8	34.06 ± 1.9
Age range (Y)	21–58	23–62
Age at onset (mean ± SD, Y)	28 ± 8.6	–
Disease Duration (mean ± SD, Y)	8.18 ± 4.1	–

Table 3 The results of Bayesian Multilevel Regression model to compare gene expression ratios between case and control groups with adjusting the effects of age and gender (*P* value estimated from Frequentist method)

Genes	Group	Controls number	Patients number	Relative expression difference (Case-Control)	SE	*P* value	95% CrI
SNHG6	Total	39	40	0.075	0.04	0.207	[−0.01, 0.15]
	Male	16	18	0.04	0.05	0.356	[−0.06, 0.13]
	Female	23	22	0.1	0.06	0.356	[−0.01, 0.2]
SNHG16	Total	39	40	0.08	0.04	0.007	[0.02, 0.14]
	Male	16	18	0.15	0.04	<0.0001	[0.08, 0.21]
	Female	23	22	0.03	0.05	0.681	[−0.07, 0.12]
LINC00346	Total	39	40	0.8	0.7	0.732	[−0.53, 2.11]
	Male	16	18	2.07	0.94	0.171	[0.2, 3.92]
	Female	23	22	−0.17	1.06	0.673	[−2.26, 1.96]
LINC00511	Total	39	40	2.28	0.72	<0.0001	[0.91, 3.67]
	Male	16	18	2.37	0.94	0.081	[0.55, 4.2]
	Female	23	22	2.21	1.1	0.006	[0.12, 4.44]

Statistical analyses

The transcript levels of genes were compared between epileptic patients and healthy subjects using Multilevel Bayesian model. Various possible distributions were fitted to the data and the final model was selected based on the WAIC and LOO indices. The observation effects were considered as random in the final model. The suitability of the model and model convergence were checked by R-hat and Gelman-Rubin diagnostics available in Shynistan. The effects of potential confounding parameters were measured through application of Quantile regression. The Box-Cox transformation was used to normalize data. The ROC (Receiver Operating Characteristic) regression model was used to measure optimal cut-off points of genes expressions for prediction of epilepsy status. The area under ROC curve (AUC), sensitivity, specificity, and optimal cut-off point were calculated according to the Youden index J (JI). The Bayesian multilevel model was estimated using Hybrid Monte Carlo with 6000 iterations and 1000 warm-up in RStan C++ library. The pROC, Stan, loo, and shynistan packages were used in R 3.5.1 environment. The statistical significance was evaluated by 95% credible interval (95% CrI) and *P* values. The level of significance was set at $P < 0.05$.

The mean values of serum vitamin D levels were compared between epileptic patients and healthy subjects using independent t-test. Cox Regression model was used for comparing the age of disease onset in epileptic patients with vitamin D deficiency and those with normal vitamin D levels. Nelson-Aalen estimator was depicted for comparison of cumulative hazard. Hazard ratio (HR) was calculated based on Exp Beta values in Cox regression model.

Results

General characteristics of study participants

Table 2 shows demographic and clinical data of patients and controls. The analysis revealed remarkable higher levels of vitamin D in healthy subjects compared with epileptic patients (Mean values of 25.76 and 31.15 for cases and controls respectively, $P = 0.003$).

Expression assays

The associations between relative expression levels of lncRNAs and epilepsy are shown in Table 3. Overall, the relative

Table 4 The results of Quantile regression model for assessment of association between relative expression of genes and independent variables

Genes	Variable	Beta	SE	t	P value	95% CI for Beta
SNHG6	Group	0.07	1.64	1.64	0.104	[−0.02, 0.14]
	Gender	−0.09	−2.26	−2.26	0.027	[−0.16, −0.01]
	Age	0.02	0.31	0.31	0.756	[−0.08, 0.1]
SNHG16	Group	0.11	2.98	2.98	0.004	[0.04, 0.18]
	Gender	−0.1	−2.67	−2.67	0.009	[−0.17, −0.03]
	Age	0.05	1.16	1.16	0.249	[−0.04, 0.13]
LINC00346	Group	0.11	0.11	0.11	0.913	[−1.77, 1.97]
	Gender	−0.43	−0.45	−0.45	0.652	[−2.29, 1.44]
	Age	1	0.95	0.95	0.345	[−1.09, 3.08]
LINC00511	Group	3.07	3.55	3.55	0.001	[1.35, 4.79]
	Gender	0.35	0.4	0.4	0.693	[−1.38, 2.06]
	Age	−0.05	−0.05	−0.05	0.961	[−1.97, 1.88]

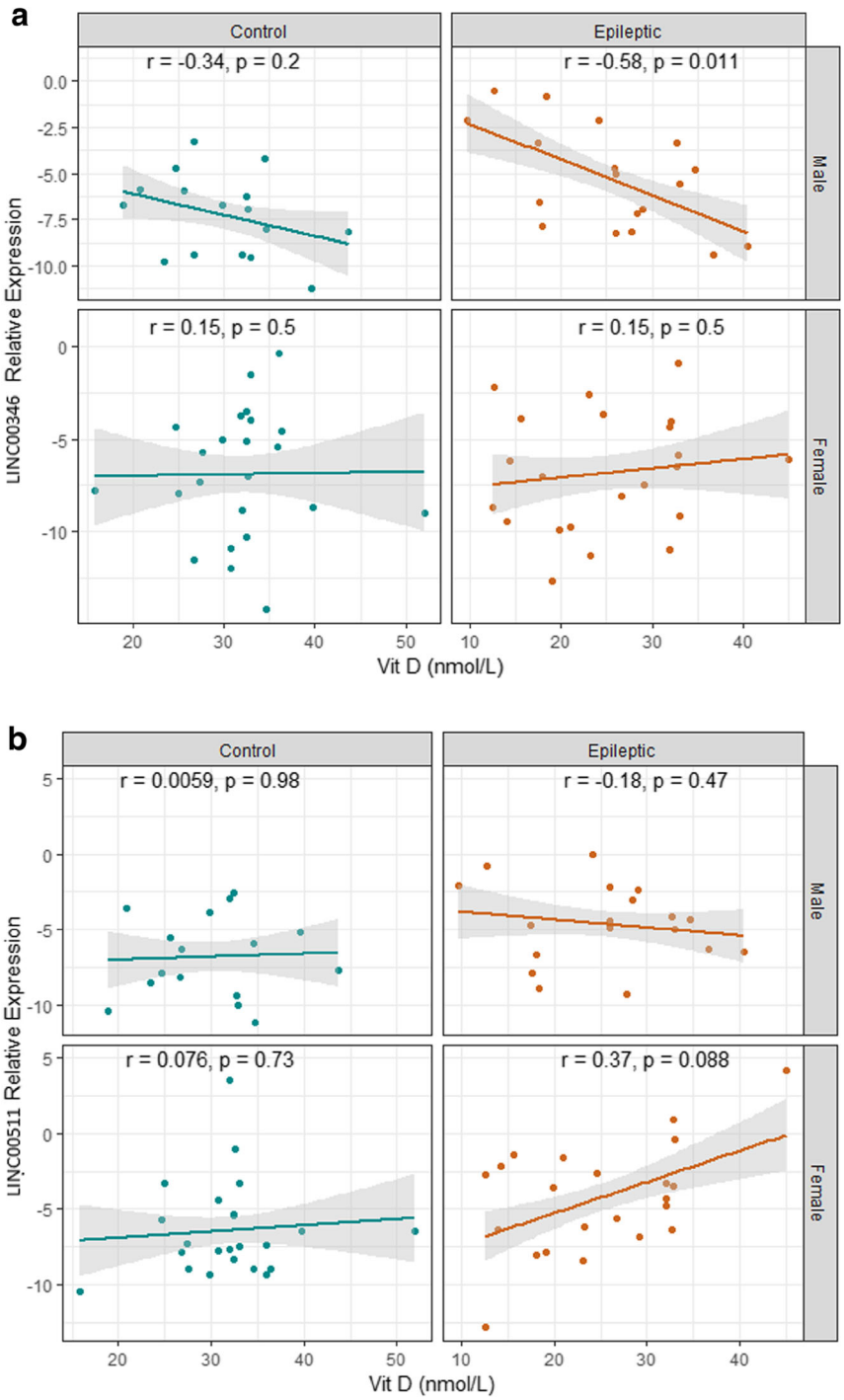

Fig. 1 Correlations between vitamin D levels and expression levels of *LINC00346* (**a**), *LINC00511* (**b**), *SNHG6* (**c**) and *SNHG16* (**d**)

expression levels of *SNHG16* and *LINC00511* were higher in epileptic patients compared with healthy subjects. For *SNHG16*, the difference was only significant between male patients and male controls while *LINC00511* had the opposite pattern.

The results of Quantile regression model for assessment of association between relative expression of genes and independent variables showed significant associations between *SNHG6* and *SNHG16* expressions and gender (*P* values of 0.027 and 0.009 respectively). Expression levels of *SNHG16* and *LINC00511* were significantly associated with disease status (*P* values of 0.004 and 0.001 respectively) (Table 4).

We also depicted the correlation matrix showing the distribution of gene expressions in total assessed individuals (Supplementary material 1). Significant correlations were detected between expression levels of *SNHG6* and *SNHG16* (r = 0.32, *P* = 0.004), *SNHG6* and *LINC00346* (r = 0.37, *P* = 0.001), *SNHG16* and *LINC00346* (r = 0.30, *P* = 0.007) as well as *SNHG16* and *LINC00511* (r = 0.29, *P* = 0.009).

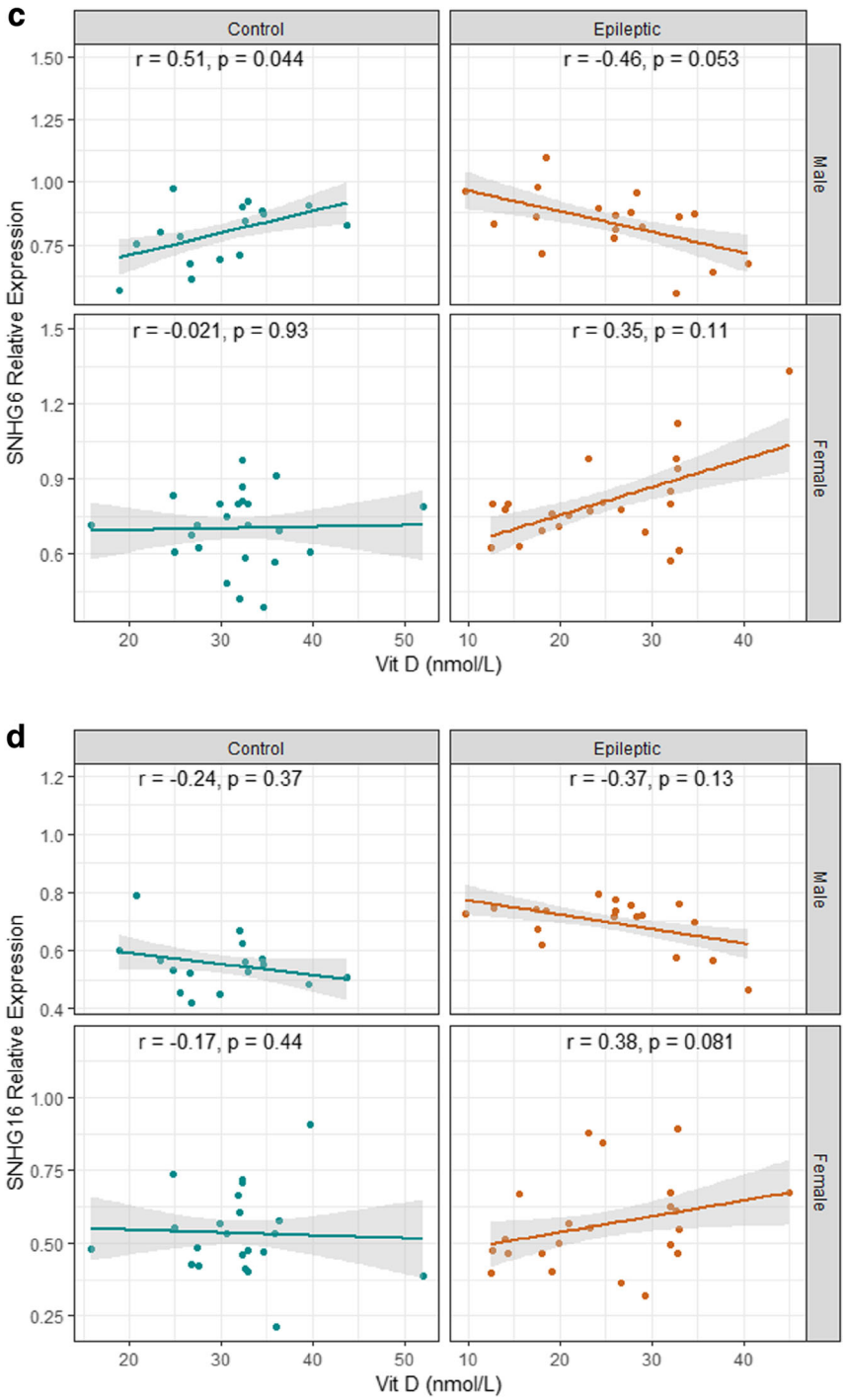

Fig. 1 (continued)

Correlation between expression levels of genes and vitamin D levels

Figure 1 shows the results of correlation analysis between expression of genes and vitamin D levels. Expression of *LINC00346* was inversely correlated with vitamin D levels only in male epileptic patients ($r = -0.58$, $P = 0.011$) (Fig. 2a). Expression of *SNHG6* was correlated with vitamin D levels in male controls but no other subgroups ($r = 0.51$, $P = 0.044$) (Fig. 2c). No other significant correlation was detected between expression of genes and vitamin D levels in any subgroup.

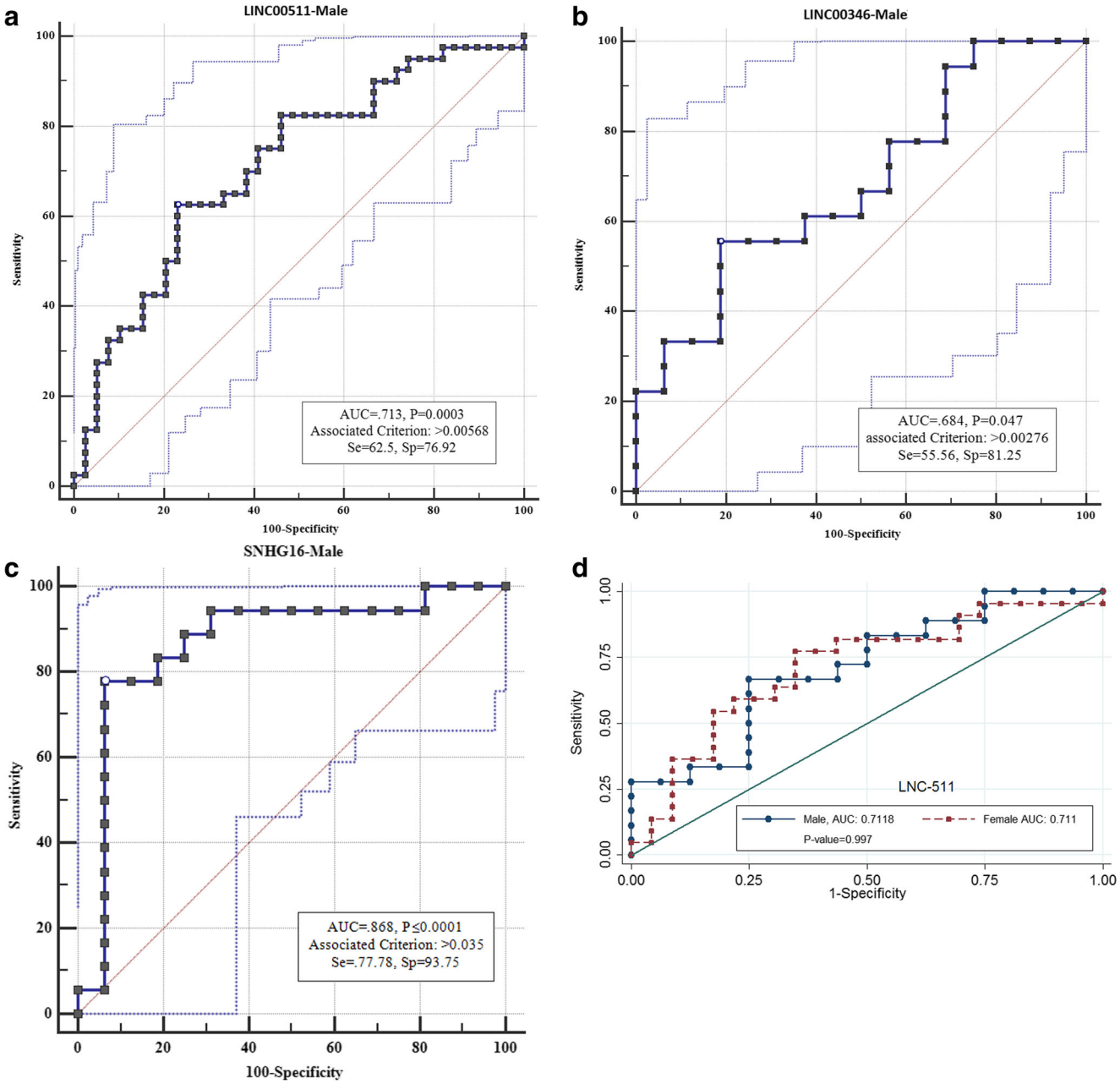

Fig. 2 ROC curve analysis of transcript levels of *LINC00511* (**a**), *LINC00346* (**b**) and *SNHG16* (**c**) in male subjects. Comparative ROC curves of *LINC00511* in males and females (**d**)

ROC curve analysis

The diagnostic power values of *LINC00511* and *LINC00346* in male subjects were estimated to be 0.71 and 0.68 respectively (Fig. 2a and b). Based on the results of ROC curve analysis, the best performance was reported for *SNHG16* in male subjects (AUC = 0.86, $P < 0.0001$) (Fig. 2c). The diagnostic power of *LINC00511* in female subjects (AUC = 0.711) was not significantly different from male subjects ($P = 0.997$) (Fig. 2d).

Assessment of cumulative hazard ratios

Nelson-Aalen estimator plots showed that male subjects with vitamin D deficiency have higher risk of disease development in earlier ages (HR = 2.96, $P = 0.044$) (Fig. 3a).

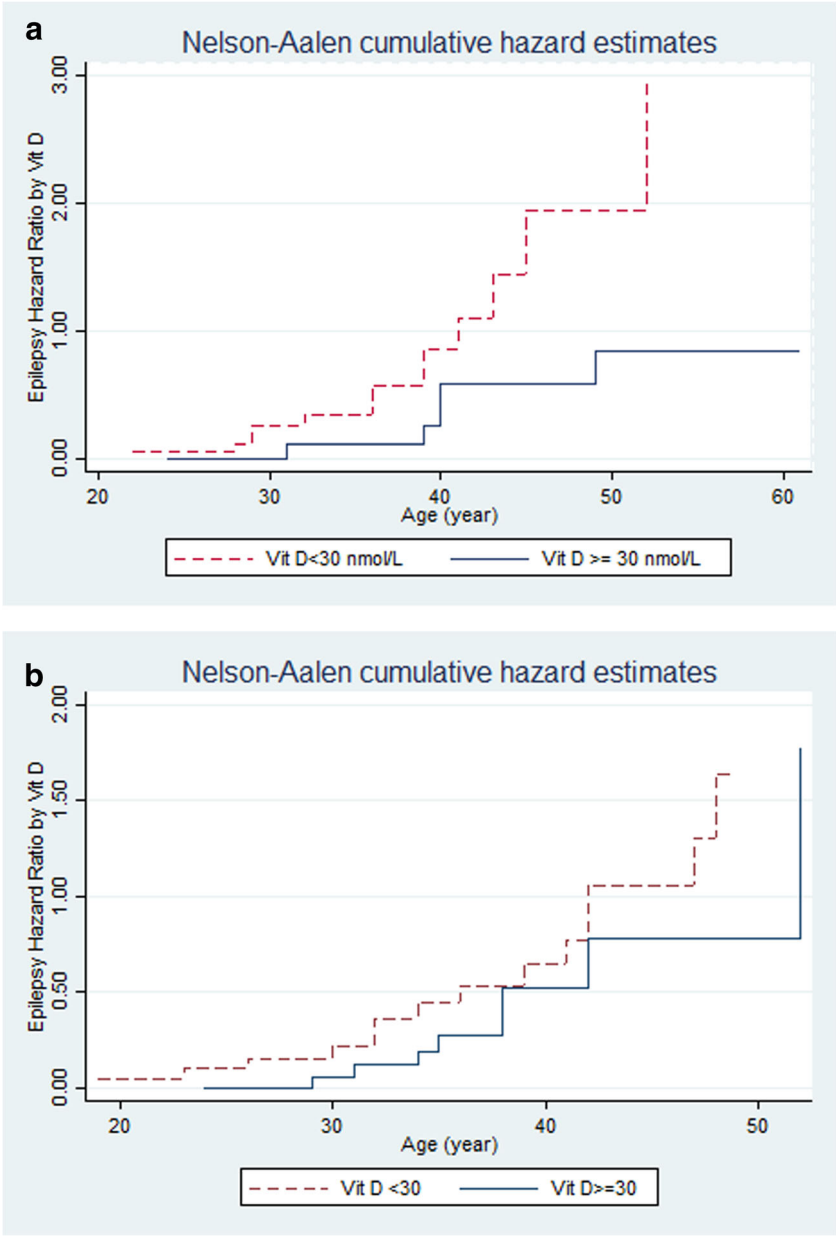

Fig. 3 Cumulative hazard ratio as depicted by Nelson-Aalen estimator plots showing the effects of vitamin D deficiency on age of epilepsy onset in male subjects (**a**) and in female subjects (**b**)

However, in female patients such effect was not significant (HR = 1.6, $P = 0.28$) (Fig. 3b).

Discussion

In the current study, we investigated expression pattern of four *VDR*-related lncRNAs in the peripheral blood of epileptic patients and healthy subjects. Vitamin D has been acknowledged as a neuroactive steroid that influences neuronal development and activity. Low serum levels of vitamin D and *VDR* polymorphisms contribute in the pathogenesis of several neurologic diseases including epilepsy (Hollo et al. 2012; Jiang et al. 2015). Although lower expression of *VDR* has been reported in epileptic patients (Mazdeh et al. 2018), the underlying mechanism of this observation has not been elucidated. Based on the acknowledged role of lncRNAs in the regulation of gene expressions and their crucial role in the central nervous system (Ng et al. 2013), we hypothesized that dysregulation of lncRNAs might be involved in the altered expression of VDR signaling pathway in the context of epilepsy. Due to the acknowledged situation for peripheral blood as an

alternative for brain tissue in gene expression analyses (Karsten et al. 2011), we assessed expression of lncRNAs in the peripheral blood of patient.

Notably, we detected higher levels of *SNHG16* and *LINC00511* expression in epileptic patients compared with healthy subjects. *SNHG16* is a small nucleolar RNA host gene with putative role in the carcinogenesis process (Wang et al. 2018). This lncRNA acts as a competing endogenous RNA for miR-15a and miR-16 (Wang et al. 2018). Notably, miR-15a and miR-16 have been recognized as targets of vitamin D in endothelial cells and breast cancer cells respectively (Zitman-Gal et al. 2014; Peng et al. 2010). In addition, *SNHG16* has interactions with miR-98 and miR-20a (Cai et al. 2017; Yang et al. 2018), other miRNAs with putative roles in vitamin D regulated pathways (Ting et al. 2013; He et al. 2010). Taken together, one can hypothesized that several non-coding RNAs are involved in construction of a complex network for regulation of VDR signaling. To add complexity to this network, *LINC00511* has been shown to inhibit expression of p57 and VEGF-A in lung and pancreatic cancer cells respectively (He et al. 2010; Zhao et al. 2018). Besides, expressions of p57 and VEGF are also regulated by vitamin D (Lu et al. 2008; Irani et al. 2017). Sex-based analyses revealed up-regulation of *SNGH16* and *LINC00511* in male and female patients compared with the corresponding control subgroups respectively. Such sex-based expression patterns imply the presence of distinct epileptogenic mechanisms in each sex. However, future studies are needed to verify this speculation.

We also demonstrated significant correlations between expression levels of *SNHG6* and *SNHG16*, *SNHG6* and *LINC00346*, *SNHG16* and *LINC00346* as well as *SNHG16* and *LINC00511*. Some of these correlations were also detected in breast tissue samples (Kholghi Oskooei et al. 2018). However, based on the calculated correlation coefficients, correlations are stronger in peripheral blood compared to breast cancer tissues.

We also compared the diagnostic power of lncRNAs and found the best performance for *SNHG16* followed by *LINC00511* and *LINC00346*. These lncRNAs might be regarded as putative biomarkers in a panel of biomarkers for diagnosis of epilepsy. We suggest assessment of expression of these lncRNAs in epileptic patients to see whether their expression levels can predict seizure occurrence.

Finally, using Nelson-Aalen estimator plots we showed that male patients but not female patients with vitamin D deficiency have higher risk of disease development in earlier ages. This finding is in accordance with the previously reported neuroprotective role of vitamin D. The observed sex-based difference further implies the presence of distinct pathogenic factors in each sex.

Taken together, our study revealed dys-regulation of certain *VDR*-related lncRNAs in the peripheral blood of epileptic patients in a sex-based manner. The main strength of our study was inclusion of a homogenous group of epileptic patients i.e. juvenile myoclonic seizures. Moreover, to decrease the effects of confounding variables, all patients were under treatment with similar dose of a single AED. However, our study has a limitation of absence of a drug-naïve group of patients to assess the possible effects of drug on gene expression.

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Compliance with ethical standards

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

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