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ORIGINAL RESEARCH

Upregulation of vitamin D-related genes in schizophrenic patients

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Introduction: Low level of vitamin D is a potential risk factor for developing schizophrenia. Through interaction with its receptor (*VDR*) and the related enzymes (*CYP27B1, CYP24A1*), vitamin D modulates neurodevelopment, neuroprotection, and immunomodulation. Its deficiency leads to aberrant neurodevelopment in schizophrenic patients.

Methods: In this case–control study, relative expression of *VDR*, *CYP27B1*, and *CYP24A1* in schizophrenic patients was compared with healthy individuals. Total RNA was extracted from whole blood of 50 patients with schizophrenia and 50 healthy controls. Real-time PCR was used to determine relative gene expression levels of *VDR*, *CYP27B1*, and *CYP24A1*.

Results: Significant upregulations were observed in *VDR* (P=0.004, 95% CI=0.77, 0.86), *CYP27B1* (P=0.002, 95% CI=1.22, 4.98), and *CYP24A1* ($P \leq 0.0001$, 95% CI=–2.721, 1.061) expressions in peripheral blood of schizophrenic patients compared with controls. Moreover, the gender-based analysis revealed upregulation of all genes in all the categories of male and female except for *VDR* gene in male group (P=0.234, 95% CI=–0.79, 3.35) and *CYP27B1* gene in the female group (P=0.09, 95% CI=–0.21, 6.55). The age-based analysis demonstrated overexpression of *VDR* and *CYP27B1* genes in all categories. Finally, there were significant correlations between expression levels of all genes ($P<0.0001$), while no correlation was found between age and expression of genes.

Conclusion: We hypothesized that the observed upregulation of the mentioned genes in schizophrenia patients might be the result of a compensatory mechanism to protect the affected individuals against adverse consequences of this disorder. Such imbalance in vitamin D processing pathway might also be implicated in the pathogenesis of schizophrenia. However, future studies should be designed to confirm the results of the current study.

Keywords: *CYP27B1*, *CYP24A1*, *VDR*, schizophrenia

Introduction

Schizophrenia is a debilitating psychiatric disorder with various etiologies affecting nearly 1% of the global population.[1] It consists of three major disabling categories: positive, negative, and cognitive symptoms.[2] Both environmental and genetic factors are involved in the etiology of this disorder.[2] Meanwhile, environmental factor might also provoke the disease onset in genetically predisposed persons. Among environmental factors, vitamin D is of particular interest. This vitamin plays a significant role in neurodevelopment as well as immunomodulation.[3] In the central nervous system (CNS), vitamin D contributes to both the synthesis of neurotransmitters and neuroprotection against damages and inflammation.[3] It is also capable of protecting the brain against reactive oxygen species (ROS), through upregulation of some known antioxidant molecules such as glutathione (GSH).[4] It has long been considered that prenatal exposure to low vitamin D may increase susceptibility to psychiatric disorders later in life. This finding is postulated by several epidemiological studies.[5] The active

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form of vitamin D is produced through two hydroxylation steps in liver and kidney. In the kidney, CYP27B1, which is a member of CYP/CYP450 superfamily is responsible for 1α hydroxylation on 25-hydroxy vitamin D_3 ($25OHD_3$) to create the active form 1α25-dihydroxyvitaminD_3 ($1\alpha25OH_2D_3$). It is worth mentioning that $1\alpha25OH_2D_3$ can regulate its production via CYP24A1 (another member of CYP/CYP450 enzymes). The latter enzyme deactivates $1\alpha25OH_2D_3$ through 24 oxidation pathway leading to the formation of calcitroic acid and lactone.[6] $1\alpha25OH_2D_3$ exerts its effects on different tissues by genomic and nongenomic pathways. Genomic pathway initiates when $1\alpha25OH_2D_3$ binds to its receptor known as VDR.[7] Thereby, it modulates the expression of several genes involved in diverse physiological functions such as calcium hemostasis, growth control, differentiation, cognition, and immune response.[8] VDR is expressed in a wide variety of nerve cells and different regions of the nervous system. Moreover, hydroxylation process is not confined to kidney and liver, but it has been discovered in numerous other tissues.[8,9] Overall, the importance of vitamin D and its metabolizing enzymes in the development of the nervous system are undeniable. Considering these and lack of studies related to the expression of genes involved in metabolism of vitamin D in schizophrenia, in this case–control study, we aimed to investigate the relative expression of *VDR*, *CYP24A1*, and *CYP27B1* in peripheral blood of schizophrenic patients and healthy controls.

Materials and methods

Study design

Clinical confirmation of schizophrenia was done according to the *Diagnostic and Statistical Manual of Mental Disorders*, Fifth Edition (DSM-V) by a certified psychiatrist. Controls were closely matched for age and gender with cases. The inclusion criteria for patients were defined as follows: definite diagnosis of the disorder based on DSM-V.[10,11] Individuals recruited in control group were assessed through a structured psychiatric interview for ruling out the presence of any psychiatric disorder. Individuals who had the drug abuse, traumatic events, and severe medical conditions such as epilepsy and history of related disorders in first-degree relatives were excluded from the study. All the patients received Clozapine™ as treatment.[12]

Blood sampling

In this study, 5 mL of peripheral blood was obtained from both groups. Written informed consent was obtained from all study participants. All procedures were performed with verification from the Ethics Committee of Shahid Beheshti University of Medical Sciences.

Laboratory analysis

Total RNA was isolated by Hybrid-RTM blood RNA extraction kit (cat no 305-101, GeneAll, Biotechnology Co. Ltd, Seoul, Korea). RNA integrity and purity were assessed by NanoDrop equipment. Low-quality RNA samples were excluded from the study. Purified RNA was stored at −80°C. In the succeeding step, the High-Capacity cDNA Reverse Transcription Kit (PN: 4,375,575, Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) was used for the synthesis of cDNA. All the procedures were performed according to the manufacturer's protocol. In order to perform TaqMan quantitative real-time PCR, the specific primers and probes were designed using Allele ID7 for x64 windows software (Premier Biosoft, Palo Alto, CA, USA). The specificity and validity of primers were determined by NCBI primer blast. The sequence of primers and probes are shown in Table S1. Finally, real-time PCR was carried out on a Corbett Rotor Gene 6000 system (Corbett Life Science, Sydney, Australia) with the TaqMan®, Universal PCR Master Mix (PN: 4,304,449, Applied Biosystems). In order to control the quality of method, we included negative control. Moreover, *HPRT1* was used as a reference gene in order to normalize relative expression of target genes. Each sample was analyzed in triplicate. Relative expression of genes in patients compared with healthy subjects was estimated based on calculation of Ln [Efficiency^(CT reference gene–CT target gene)] values.

Statistical analysis

The independent sample *t*-test was applied to examine the differences between two groups. The one-way ANOVA test was used to compare means between control and patient groups. Pearson's correlation coefficient was calculated to assess correlations between variables. The Kolmogorov–Smirnov test was used to investigate the normality. The level of significant *P*-value was set at ≤ 0.05. Data analysis was performed using SPSS version 18 (Chicago, IL, USA).

Result

Clinical and demographic information

The demographic features of the study groups are summarized in Table 1. No significant difference was found in age of cases and controls ($P=0.0581$) or in sex ratio ($P=0.18$). All the individuals were compared in three ways: A) total participants together (regardless of age and sex), B) sex-based analysis (male or female), and C) age-based analysis

Table 1 Demographic features of schizophrenia patients and healthy controls

Variables	Schizophrenia patients	Control
Female/male, n (%)	15 (30)/35 (70)	18 (36)/32 (64)
Age (years), mean ± SD	50.7±4.2	49.2±3.6
Age range (years)	30–69	29–63
Male <30	7	8
Female <30	0	4
30< male <40	13	8
30< female <40	4	7
Male >40	15	16
Female >40	11	7
Age at onset (years), mean ± SD	35±1.2	–
Years of illness, mean ± SD	8±0.04	–
Education		
Preschool (%)	30	12
School (%)	48	28
University (%)	22	60

(<30, 30–40, and >40 years). Therefore, the group of schizophrenia patients was compared with controls, as well as each of the sex and age subgroup.

Relative expression level of the *VDR*

We observed upregulation of *VDR* expression in the peripheral blood of schizophrenic patients compared with healthy controls (P=0.004, 95% CI=0.77, 0.86). In the gender-based analysis of *VDR* expression, we detected a statistically significant change in expression levels of VDR in female affected by schizophrenia (P<0.0001, 95% CI=3.17, 6.15). Furthermore, schizophrenic females aged between 30 and 40 years and both male and female patients over the age of 40 years had a significant increase in expression of *VDR* (P=0.001, 95% CI=4.25, 7.09; P=0.047, 95% CI=0.03, 6.36; and P=0.03, 95% CI=1.15, 6.83, respectively) (Table 2).

Relative expression level of the *CYP27B1*

CYP27B1 total expression level was significantly higher in the peripheral blood of schizophrenic patients compared with controls (P=0.002, 95% CI=1.22, 4.98). Gender-based assessments revealed upregulation in both male and female groups. However, this increase was merely significant in the male group (P=0.012, 95% CI=0.7, 5.41). In addition, we observed *CYP27B1* upregulation in schizophrenic male patients aged over 40 years compared with the corresponding control group (P=0.01, 95% CI=1.32, 8.7) (Table 2).

Relative expression level of the *CYP24A1*

CYP24A1 expression level was increased in the peripheral blood of schizophrenic patients compared with controls

Table 2 Expression levels of genes in schizophrenia patients compared with healthy controls

	Control number	Patient number	*VDR*				*CYP27B1*				*CYP24A1*			
			Expression ratio	SE	*P*-value	95% CI	Expression ratio	SE	*P*-value	95% CI	Expression ratio	SE	*P*-value	95% CI
Total	50	50	3.2279	0.774	0.004	0.77, 0.86	5.8362	0.94	0.002	1.22, 4.98	1.9307	0.421	<0.0001	−2.721, −1.061
Male	32	35	1.8859	1.031	0.234	−0.79, 3.35	6.1354	1.17	0.012	0.7, 5.41	2.111	0.564	0.003	−2.819, −0.577
Female	18	15	10.6948	0.727	<0.0001	3.17, 6.15	4.955	1.67	0.09	−0.21, 6.55	1.8357	0.897	0.042	−4.42, −0.93
Male <30 years	8	7	0.4959	2.6	0.608	−6.6, −3.7	0.6075	1.87	0.6	−5.12, −2.52	0.9016	2.404	>0.999	−3.773, 5.841
Female <30 years	4	0	1.7502	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Male 30–40 years	8	13	1.3523	1.47	0.781	−2.55, 3.3	3.1416	1.94	0.271	−1.181, 6.02	1.3076	1.267	0.171	−3.731, 1.396
Female 30–40 years	7	4	18.8422	0.73	0.001	4.25, 7.09	25.3907	2.66	0.09	−0.66, 11.19	1.2352	−3.325	0.073	1.87, 7.29
Male >40 years	16	15	4.7965	1.54	0.047	0.03, 6.36	4.7965	1.79	0.01	1.32, 8.7	0.9132	−1.608	0.002	−0.679, 3.064
Female >40 years	7	11	7.7907	1.38	0.03	1.15, 6.83	2.8732	2.17	0.37	−1.91, 6.58	1.0365	−1.863	0.044	−0.421, 2.721

Abbreviation: NA, no analysis.

($P<0.001$, 95% CI=–2.721, –1.061). Such upregulation was also significant in male and female subgroups ($P=0.003$, 95% CI=–2.819, –0.577 and $P=0.042$, 95% CI=–4.42, –0.93, respectively). However, in the subgroup of patients aged over 40 years, both male and female patients demonstrated downregulation of *CYP24A1* expression ($P=0.002$, 95% CI=–0.679, –3.064 and $P=0.044$, 95% CI=–0.421, –2.721, respectively) (Table 2).

Correlation between the expression levels of *VDR/CYP27B1*, *VDR/CYP24A1*, and *CYP27B1/CYP24A1* in schizophrenic patients and healthy controls

Correlations between expression levels of *VDR/CYP27B1*, *VDR/CYP24A1*, and *CYP27B1/CYP24A1* are demonstrated in Figure 1A–C. All correlations were statistically significant ($P<0.0001$).

Correlation between expressions of vitamin D-related genes and age of study participants

Increase in the age of schizophrenia patients did not affect the expression level of the vitamin D-related genes. As in Figure 2A–C, there were not any statistically significant correlation between expression of genes and age either in patients or healthy subjects ($P>0.05$).

Discussion

In this study, we compared the expression of vitamin D-related genes in schizophrenic patients and healthy controls using quantitative real-time PCR. Our data revealed significant upregulation of *CYP27B1*, *VDR*, and *CYP24A1* in schizophrenia patients compared with controls ($P=0.002$, $P=0.004$, and $P<0.0001$, respectively). In order to examine the clinical relevance of our results, we also assessed the expression of these genes in brain samples using the public available data of the SZDB database (http://www.szdb.org/). The dataset GSE12649 showed upregulation of *VDR* (fold change = 1.54, $P=0.047$ with 204255_s_at probe). However, expressions of the other genes were not different between cases and controls using the same dataset.

In schizophrenia, males are more affected than females. Several lines of evidence suggest that estrogen in female can have a protective role against neuropsychiatric diseases. Both estrogen and vitamin D activate TPH_2 gene responsible for serotonin production.[13–15] Therefore, we hypothesized that the change in expression ratio of VDR and CYP27B1 is more probable in male than female or it is plausible that male display alteration in vitamin D gene expression with more intensity. As a result, we carried out gender-based analysis. However, in contrast to our speculation, female

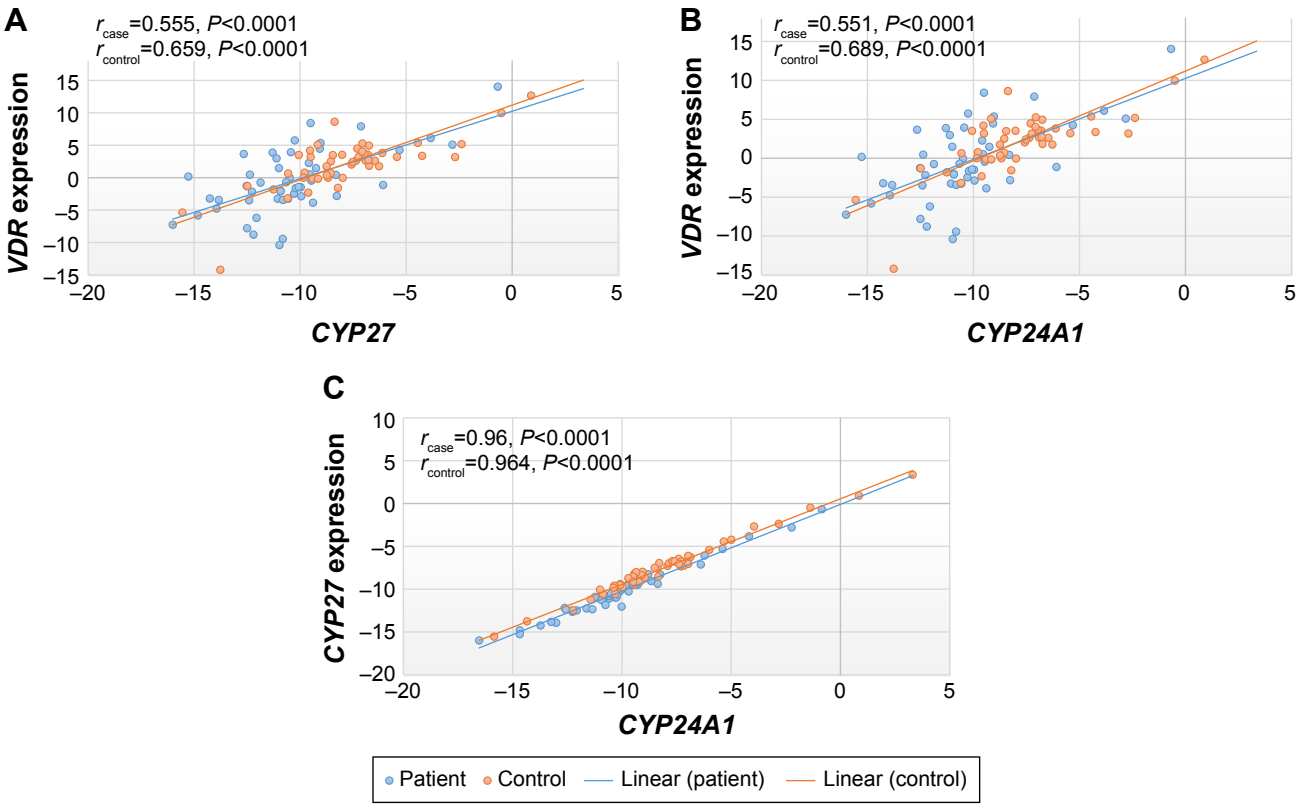


Figure 1 (**A**) Correlation between *VDR* and *CYP27* expressions. (**B**) Correlation between *VDR* and *CYP24A1* expressions. (**C**) Correlation between *CYP27* and *CYP24A1* expressions.

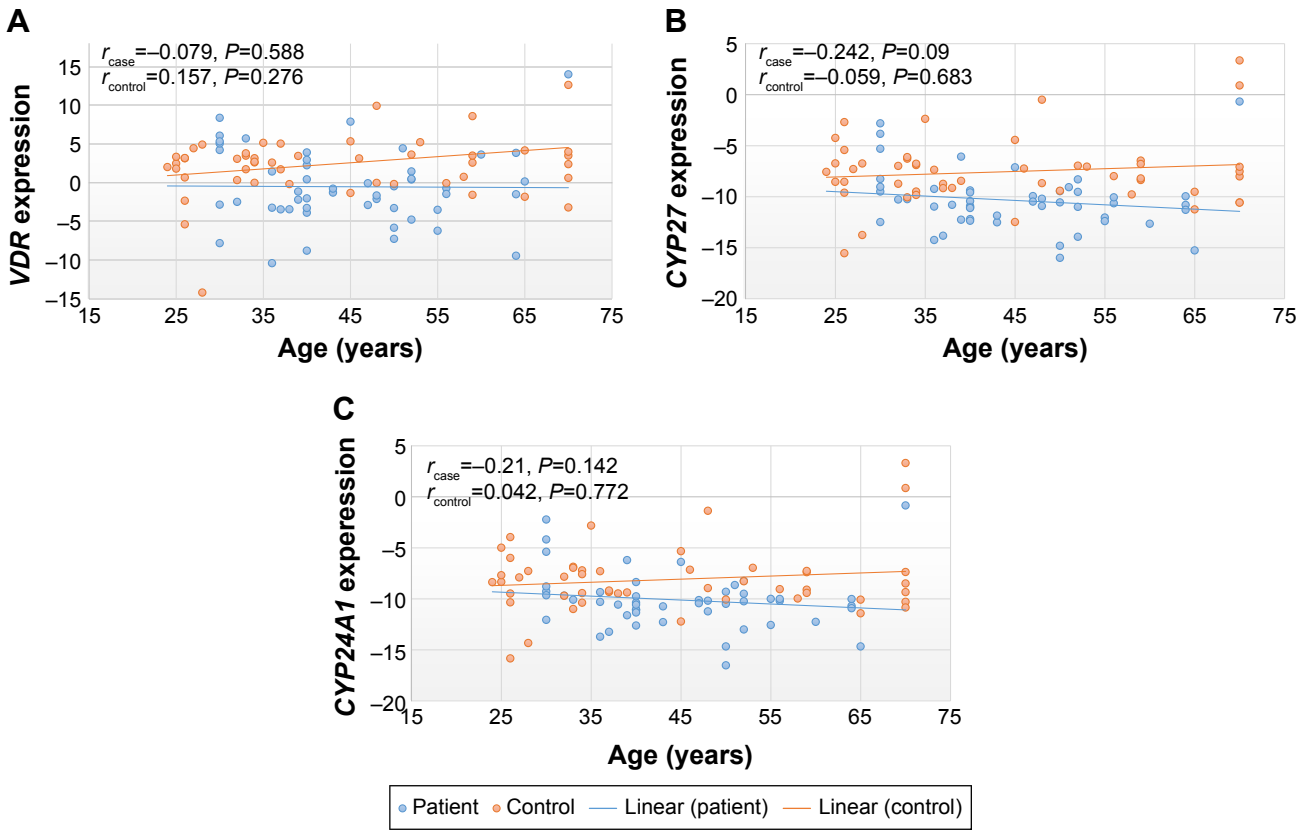


Figure 2 (**A**) Correlation between *VDR* expression and age. (**B**) Correlation between *CYP27* expression and age. (**C**) Correlation between *CYP24A1* expression and age.

patients revealed a significant increase in *VDR* expression ($P<0.0001$). Furthermore, in *CYP27B1* expression analysis, both male and female patients had considerable upregulation compared with healthy controls. However, only *CYP27B1* upregulation in male category was statistically significant ($P=0.012$). In the age-based analysis, based on estrogen hypothesis that was mentioned earlier, we expected upregulation in *VDR* and *CYP27B1* gene expression in affected female over 40 years old because these individuals have lower amounts of estrogen. For *VDR* gene, these results were not compatible with the suggested hypothesis. For instance, both female and male over 40 years old showed increase in the *VDR* expression ($P=0.03$ and $P=0.04$, respectively), while female aged between 30 and 40 years also represented this increase ($P=0.001$). *CYP27B1* overexpression was only significant for schizophrenic male patients aged over 40 years ($P=0.01$). *CYP24A1* gene revealed downregulation in both male and female patients aged over 40 years ($P=0.002$ and $P=0.004$, respectively). To our knowledge, this was the first study evaluating the expression levels of VDR and related enzymes in schizophrenia. So far, most of the studies that have assessed vitamin D-related gene in schizophrenia have mainly focused on polymorphisms of these genes[16,17] or assessed serum level of vitamin D in schizophrenic patients and reported lower serum level of vitamin D in these patients compared with controls.[18] In this study, we also assessed the correlation between the expression of vitamin D-related genes and the age of study participants. Expressions of the assessed genes were not correlated with age either in schizophrenia patients or in controls, which is in line with the findings reported in multiple sclerosis patients.[19] On the contrary, all pairwise correlations between expression levels of genes were significant. In our study, schizophrenia patients had been treated with an equal dose of clozapine. Clozapine is an atypical drug, and its mechanism of action is not comprehensively known. It acts on histaminergic, muscarinic, adrenergic, and serotonin receptors. Gene expression studies using microarray in both rat and human postmortem brain showed that clozapine regulates several genes acting in various biological pathways. Genes that are modulated by this drug are suggested as candidate genes in schizophrenia. However, vitamin D signaling pathway was not among those possibly regulated pathways by clozapine.[20,21] This may indicate that changes in vitamin D in schizophrenia can be the consequence of this disorder not the cause of it. Moreover, clozapine does not obviate the action of DHCR7 enzyme and does not alter the level of vitamin D precursor (7DHC) either in rat or in human.[22] Overall, it is unlikely that change

in *VDR*, *CYP24A1*, and *CYP27B1* expression observed in schizophrenia patients in this study is the result of clozapine action on gene regulation. Our findings suggest that increased expression levels of *VDR* and *CYP27B1* may be involved in the pathophysiology of schizophrenia through various pathways. There are growing evidences linking vitamin D to neuroplasticity, neuroprotection, and neurotransmission. Low level of this vitamin is considered as a potential risk factor for developing psychiatric and neurological disorders.[9] Vitamin D deficiency can cause long-term dysregulation in genes that are involved in mitochondrial function, cytoskeletal maintenance, and neurotransmission.[5] Previous reports indicate the presence of vitamin D metabolite 25(OH)D in the cerebrospinal fluid (CSF) and the correlation between CSF and serum 25(OH)D levels.[23] Besides, vitamin D and its carrier protein infiltrate to the CNS.[24] So, we suggest that the expression of metabolizing enzymes in the blood might affect vitamin D levels in the brain. However, future studies are needed to verify this hypothesis.

As mentioned earlier, schizophrenia is a disorder of heterogeneous nature. Several studies have concentrated on the dopaminergic hypothesis of schizophrenia. Nevertheless, in recent years, growing number of studies have focused on other aspects of this disorder such as neuroinflammation and redox dysregulation along with NMDA receptor (NMDAR) hypofunction. Redox dysregulation is observed in schizophrenia patients.[25] The imbalance in the production of ROS and reactive nitrogen species along with deficiency in enzymatic and nonenzymatic antioxidants can lead to oxidative and nitrosative stress.[25] GSH is a cellular antioxidant. Its synthesis and metabolism are controlled by gamma-glutamyl transpeptidase (GGT). It is now postulated that the active form of vitamin D (1,25OH2D3) upregulates activation and gene expression of GGT. Furthermore, 1,25OH2D3 increases activation of GSH reductase, GSH peroxidase, and glutamyl cysteine ligase catalytic subunit.[26–29] Vitamin D serum level can elevate GSH expression in astrocytes. Additionally, diminished levels of GSH were observed in prefrontal cortex and CSF of schizophrenia patients and its lower levels associated with more severe negative symptoms.[2,26–28,30] Interestingly, this antioxidant agent has the capability to regulate different neurotransmitter systems such as NMDARs and GABA-A receptor. GSH deficiency as a result of redox dysregulation can lead to NMDAR hypofunction and decreased myelination

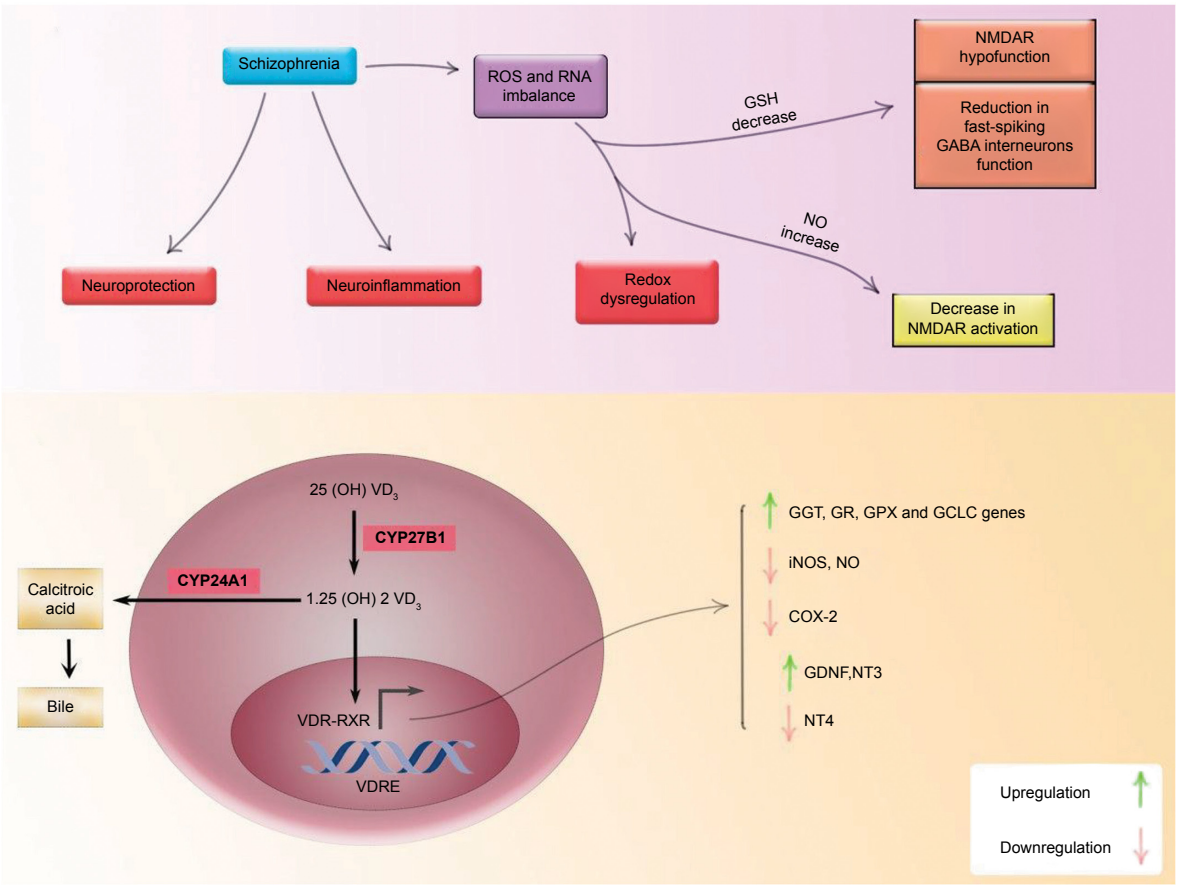


Figure 3 The role of vitamin D in four pathways involved in the etiology of schizophrenia.

in schizophrenia patients, two conditions that can reduce fast-spiking GABA interneurons function causing negative, positive, and cognitive symptoms of schizophrenia.[25,31] Based on the available evidence, low level of GSH can be a potential factor for NMDAR hypofunction, which is reported in schizophrenia. 1,25OH2D3 also acts as a neuroprotective agent through downregulation of inducible nitric oxide synthase (iNOS).[2,30] This enzyme releases nitric oxide (NO) during the reaction. NO metabolites damage neurons as well as oligodendrocytes when they are produced in large amount. In postmortem studies, elevated levels of NO and NOS was identified in schizophrenic patients.[26,32] There is also evidence indicating immune activation in schizophrenia. Inflammatory symptoms have been observed in postmortem studies in schizophrenia patients. As a result, inflammation might be a possible pathogenic mechanism in this disorder. COX2 (cyclooxygenase 2 is a rate-limiting enzyme in prostaglandin biosynthesis) mediates inflammatory response, and it is induced by cytokines such as IL2, IL6, and IL10. By recruiting genomic pathway, 1,25OH2D3 suppresses transcription of COX2 gene, which can lead to the succeeding inhibition of proinflammatory cytokines secretion.[33,34] On the contrary, 1,25OH2D3 works as a neurotrophic support through upregulation of GDNF (glial cell line-derived neurotrophic factor), NT3 (neurotrophin 3), and downregulation of NT4 (neurotrophin 4).[35] Figure 3 has summarized the role of vitamin D in four pathways involved in the etiology of schizophrenia. CYP27B1 converts vitamin D to its active form 1,25OH2D3, which is then bound to its receptor VDR. VDR heterodimerized with retinoid × receptor and modulates the expression of target genes. As mentioned above, it elevates GSH levels and upregulates mRNA expression of NGF, NT3, GDNF, and GGT, while it downregulates inflammation and mRNA expression of iNOS, COX2, and NT4 in astrocytes and microglia. CYP24A1 degrades this active form and terminates its effects.[30] In this study, we observed approximately two-fold increase in CYP24A1 expression level, which can be justified by positive feedback through VDR as a result of increase in CYP27B1. Moreover, we found that patients with schizophrenia had significant upregulation in *VDR* and *CYP27B1* gene expression. Therefore, we hypothesized that these findings might be a consequence of schizophrenic disorder in order to apply the compensatory mechanism to promote antioxidant protection against oxidative and nitrosative stress as well as alleviating neuroinflammation and boosting neurotrophic support in patients affected by schizophrenia. Finally, the main limitation of this study is the lack of first-episode schizophrenia patients in order to evaluate the gene expression free of possible confounding effects of medication. Moreover, we did not have information about vitamin D status of study participants, and we did not have access to postmortem brain tissues to evaluate expression of genes in them.

Conclusion

Schizophrenia is a severe mental condition with a diversified set of pathologies such as perturbed dopamine and glutamate pathways, aberrant redox regulation, and neuroinflammation, all of which create a common central pathway.[29] The active form of vitamin D upregulates or downregulates numerous factors acting in these pathways. Both VDR coding mRNA and its protein are present in the CNS. The mRNA expression of VDR gene has been identified both in neuronal and glial cells. Our findings suggest that increased mRNA expression of *VDR* and *CYP27B1* may be involved in the modulation of mentioned pathways through the genomic mechanism in order to ameliorate symptoms of the disease. Further investigations are needed to support this claim and determine the exact regulatory mechanism of vitamin D in each pathway. It is also valuable to measure the expression levels of VDR and related enzymes simultaneously with that of other molecular agents involved in oxidative, nitrosative stress, immune activation, and neurotrophic support in schizophrenia patients.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary material

Table S1 The sequences of probes and primers

Property	*VDR*	*HPRT1*
F-primer	TGGCTTTCACTTCAATGCTATGA	AGCCTAAGATGAGAGTTC
R-primer	CGTCGGTTGTCCTTGGTGAT	CACAGAACTAGAACATTGATA
Probe	FAM-ACTTCCGGCCTCCAGTTCGTATGGAC-TAMRA	FAM -CATCTGGAGTCCTATTGACATCGC- TAMRA
Property	***CYP27B1***	***CYP24A1***
F-primer	CCCAGATCCTAACACATTTTGAGG	TATCGCGACTACCGCAAAGA
R-primer	AAAGGGTGATGATGACAGTCTCTTTC	CGGCCAAGACCTCATTGATT
Probe	FAM-ACCCAAGACCCGGACTGTCCTGGT –TAMRA	FAM-TCCGGACCCGCTGCCAGTCTT –TAMRA

Abbreviations: F, forward; R, reverse.

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