Association between vitamin D metabolism gene polymorphisms and schizophrenia

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Abstract. Schizophrenia (SZ) is a multifactorial and neurodegenerative disorder that results from the interaction between genetic and environmental factors. Notably, hundreds of single nucleotide polymorphisms (SNPs) are associated with the susceptibility to SZ. Vitamin D (VD) plays an essential role in regulating several genes important for maintaining brain function and health. To the best of the authors' knowledge, no studies have yet been conducted on the association between the VD pathway and patients with SZ. Therefore, the present study aimed to assess the potential association between eight SNPs in genes related to the VD pathway, including *CYP2R1*, *CYP27B1*, *CYP24A1* and *VDR* among patients with SZ. A case-control study was conducted, involving a total of 400 blood samples drawn from 200 patients and 200 healthy controls. Genomic DNA was extracted and variants were genotyped using the tetra-amplification refractory mutation system-polymerase chain reaction method. The present study revealed statistically significant differences between patients with SZ and controls regarding the genotypes and allele distributions of three SNPs [*CYP2R1* (rs10741657), *CYP27B1* (rs10877012) and *CYP24A1* (rs6013897) (P<0.0001)]. The AA genotype of rs10741657 was identified to be associated with SZ (P<0.0001) and the frequency of the A allele was higher in patients with SZ (P<0.0001) compared with the control group. Similarly, the TT genotype of rs10877012 was revealed to be associated with SZ (P<0.0001) and the T allele was more frequent in patients with SZ (P<0.0001) than in the control group. Moreover, the AA genotype of rs6013897 was revealed to be associated with SZ (P<0.0001), although no significant difference was detected between the two groups regarding the A allele (P=0.055). *VDR* (rs2228570, rs1544410, rs731236 and rs7975232) and *CYP27B1* (rs4646536) gene polymorphisms did not exhibit a significant association with SZ. While the studied SNPs revealed promising discriminatory capacity between patients with SZ and controls, the rs10741657 SNP exhibited the most optimal area under the curve value at 0.615. A logistic model was applied considering only the significant SNPs and VD levels, which revealed that rs6013897 (T/A) and VD may have protective effects (0.267, P<0.001; 0.888, P<0.001, respectively). Moreover, a low serum VD level was highly prevalent in patients with SZ compared with the controls. Based on this finding, an association between serum 25(OH)D and SZ could be demonstrated. The present study revealed that *CYP2R1* (rs10741657), *CYP27B1* (rs10877012) and *CYP24A1* (rs6013897) gene SNPs may be associated with SZ susceptibility.

Introduction

Mental disorders are considered a global public health issue and continue to be a major burden worldwide (1). As stated by the National Institute of Mental Health, these disorders mainly affect the mentality, behavior or emotions of an individual, and can vary in terms of impairment degree, significantly influencing their activities, education, employment and social participation. Among the mental disorders that can lead to psychosis is schizophrenia (SZ), which is considered one of the most severe and debilitating psychiatric disorders with a mean lifetime prevalence of ~1% of the population (2). This percentage varies according to ethnicity, culture and geographical area (3). In the Arab world, in particular, several studies have reported that SZ affects 0.7-5.6% of the population (4). Recurrent episodes of psychosis characterized by hallucinations, delusions and cognitive impairment represent the primary symptoms of the disease. These symptoms can vary across patients and throughout the course of the disease (5). Genetically, there is considerable evidence suggesting that SZ has a heritability rate of 66-85% (6); the remaining influence may be attributed to environmental factors. Nevertheless, the exact mechanism by which gene-environment interactions influence the susceptibility to SZ remains unclear.

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Genome-wide association studies have traditionally been used to investigate various complex disorders (7-11). Previous association studies have aimed to identify genetic variations, known as single nucleotide polymorphisms (SNPs), within genes with known neurological functions and to investigate their contribution to the development of SZ (7,10,11). Various effects of these variants have been reported, and both common and rare variants have been associated with either large or small individual susceptibility to the disease (12-15). These variants could influence the expression of genes involved in brain development, providing researchers with valuable insights into the brain dysfunction underlying the symptoms of SZ. The neurodevelopmental hypothesis of SZ previously proposed that the interaction between genetic and environmental factors, such as vitamin D (VD) deficiency, can alter brain function during early critical phases of brain development, causing brain impairment and dysfunction (16-19). VD is considered a neurosteroid regulator that exerts its action through binding to the VD receptor (VDR) in numerous tissues, including the brain (20,21). Therefore, VD is essential for proper neurodevelopment, and cognitive and behavioral function.

Based on the previous literature, VD imbalance has been associated with the development of numerous psychiatric disorders, including SZ (20,22,23). Accordingly, a number of studies have investigated the genetic determinants of this hormone (24-27). Research has aimed to determine whether specific genetic variations in VD metabolism genes are associated with VD levels or VD-related health outcomes. In the present study, eight SNPs were investigated within VD metabolism-related genes to assess their potential association with SZ susceptibility in Jordanian patients. These SNPs included rs10741657, a 5' UTR A/G substitution associated with altered enzyme activity of *CYP2R1* and hypovitaminosis D, where the GG genotype is linked to decreased [25(OH)D] levels compared with the AA genotype (28,29). Additionally, the *CYP27B1* SNPs rs10877012 (G>T) and rs4646536 (A>G) have been reported to influence circulating calcitriol serum levels and to be associated with type 1 diabetes (30). Furthermore, the rs6013897 SNP located at the 3' flanking region of the *CYP24A1* gene has been revealed to be positively associated with circulating [25(OH) D] levels (31). Other SNPs distributed across the *VDR* gene, such as rs2228570, rs1544410, rs731236 and rs7975232 (32-34), have been extensively studied and were revealed to affect gene expression and VDR protein levels, with variable levels of distribution across ethnicities and sexes. These SNPs have been associated with neurodevelopmental and neuropsychiatric disorders, including SZ, mental health disorders and autism, although the findings are conflicting. In order to create a panel of key SNPs linked to SZ, the present study intended to identify how these studied SNPs, which were selected based on their known influence on VD metabolism and their association with neurological disorders, might be associated with SZ susceptibility in a group of Jordanian patients with SZ.

Materials and methods

Study participants. A total of 400 subjects were enrolled in the present study and divided into two groups: i) 200 patients diagnosed with SZ attending a psychiatric clinic at King Abdullah University Hospital and Princess Basma Teaching Hospital (Irbid, Jordan); ii) 200 healthy controls free of any psychosis-related symptoms attending the National Center for Diabetes Endocrinology and Genetics (Amman, Jordan) for routine health care. The present study was approved by the Institutional Review Board Committee (approval no. 2019/626) of Jordan University of Science and Technology (Irbid, Jordan) and written informed consent was obtained from all participants before enrollment in the present study.

Sample collection. After collecting the consent forms signed by all of the enrolled patients with SZ and controls, two peripheral blood samples were collected in plain (5 ml) and EDTA (4 ml) tubes. The samples were collected between May 1, 2020 and September 30, 2021. Serum was separated after centrifuging blood samples in plain tubes at 10,000 x g for 10 min at 4˚C and was stored at -80˚C for later use to measure VD concentration. Notably, hemolyzed samples were excluded from the present study. Blood samples in EDTA tubes were used for DNA extraction and further analysis.

Inclusion and exclusion criteria. Patients were diagnosed by a proficient psychiatrist according to the diagnostic criteria for SZ based on the ICD-10 (DSM-V) (35). Experienced psychiatrists conducted these diagnoses, following standard guidelines to ensure accuracy. Patients were treated according to the latest best practices and medical standards as outlined by the guidelines issued by the American Psychiatric Association. The participants who met the following criteria were included: i) Either sex between 18-60 years old; ii) sporadic and familial cases; iii) no history of other mental disorders; iv) no history of blood transfusion within 1 month; v) no physical and nervous system diseases, such as brain trauma; vi) no history of alcohol abuse and drug abuse. The exclusion criteria were: i) Presence of other medical conditions, which may produce psychotic SZ-related symptoms, such as epilepsy, metabolic disturbance, brain lesions, limbic encephalitis, stroke, multiple sclerosis and dementia; ii) presence of other diseases or medications known to affect VD, such as arthritis, osteoporosis, end-stage renal disease, hypothyroidism, rickets, corticosteroid therapy and malabsorption syndromes; and iii) individuals with drug-induced psychosis, acquired brain injuries and intellectual disabilities.

Genomic DNA extraction. Genomic DNA was extracted using the QIAamp DNA Mini Kit (cat. no. 51304; Qiagen GmbH) according to the manufacturer's instructions. DNA concentration was measured using a Nanodrop (Thermo Fisher Scientific, Inc.) and integrity was verified using 2% agarose gel electrophoresis.

SNP selection and genotyping. For SNP genotyping, two sets of allele-specific primers for each SNP were designed using the PRIMER1 tool (http://primer1.soton.ac.uk/primer1.html). Tetra-amplification refractory mutation system-polymerase chain reaction (T-ARMS-PCR) was carried out for rs10741657, rs10877012, rs4646536, rs6013897, rs2228570, rs1544410, rs731236 and rs7975232 genotyping. The primer sequences are listed in Table SI. The annealing temperature for each primer

set was optimized through gradient PCR that was carried out on a Veriti™ Dx 96-well Thermal Cycler (Thermo Fisher Scientific, Inc.). Each PCR was carried out in a total volume of 20 μl, containing 4 μl HOT FIREPol® Blend Master Mix (cat. no. 04-27-00115; Solis BioDyne), 0.5 μl each primer, 1 μl DNA template and 13 μl nuclease-free water. The PCR cycling conditions were as follows: Initial denaturation at 95˚C for 10 min, followed by 35 cycles of denaturation at 95˚C for 15 sec, annealing at 61-64˚C for 30 sec, extension at 72˚C for 2 min, and a final extension at 72˚C for 10 min. Subsequently, 10 μl PCR products were loaded onto a 3% agarose gel, and a 100-bp ladder (cat. no. MBT049; HiMedia Laboratories) was used for size comparison between DNA fragments. For gel visualization, a UV transilluminator (Cleaver Scientific Ltd.) was used.

VD concentration measurement. Serum 25(OH)D levels were determined using the commercially available Roche Elecsys Vitamin D total II electrochemiluminescence immunoassay and Cobas E801 auto analyzer (Roche Diagnostics GmbH).

Statistical analysis. Statistical analysis was carried out using SPSS software (Version 22; IBM Inc.). The genotype frequencies of all SNPs were compared using a χ^2 test or Fisher's exact test when >20% of expected cell counts are <5. Logistic regression analysis was performed to determine the odds ratio (OR) and 95% confidence interval associated with SZ risk, with the control considered the reference group. Each polymorphism was tested for Hardy-Weinberg equilibrium (HWE) using χ^2 test or Fisher's exact test. $P<0.05$ was considered to indicate a statistically significant difference.

Moreover, a total of 370 cases were included for the receiver operating characteristic (ROC) curve analysis after excluding individuals with missing genotype data for any of the investigated SNPs. For the generalized linear model, the analysis was conducted on a subset of 251 cases due to a lack of VD measurement data or genotype data for one of the studied SNPs. ROC curve analysis was performed using the classify module in SPSS software, encompassing all studied SNPs, to predict the presence of SZ. Subsequently, the generalized linear model was employed, utilizing binary logistic regression in the type of model menu. The dependent variable in the response menu was set as the status of the samples, referencing the control samples. The predictor menu included the three studied SNPs as factors, while VD levels and age served as covariates. The wild-type genotype for each SNP was designated as the baseline category, coded as 1. Within the model menu, the main effect for all SNPs and VD was specified. Finally, the likelihood ratio test with profile likelihood was utilized for model evaluation. These comprehensive methods were implemented to investigate the predictive utility of the included SNPs through ROC curve analysis and to explore the association between genetic variants, VD levels, age and SZ status using generalized linear modeling techniques.

Furthermore, SRplot (http://www.bioinformatics.com.cn/en) was used to perform Mann-Whitney U test when comparing VD levels between patients with SZ and controls, which included 272 cases in total. The effect of sex variation between groups on the genotyping frequency was assessed using Pearson's χ^2 test or Fisher's exact test. Linkage disequilibrium (LD) analysis was conducted using the SNP linkage LD heatmap module available on SRplot (http://www.bioinformatics.com.cn/srplot). This module calculates pairwise LD statistics, measured by R^2, between SNPs. These statistical data are visually represented in a triangular heatmap, where the extent of LD between SNP pairs is indicated through a color-coded scale. The color key is used to denote R^2 values, enhancing the visual interpretation of LD strength. Additionally, the heatmap integrates gene models with SNP sites marked by colored asterisks, providing a clear genetic landscape and facilitating the identification of regions with strong LD. This graphical representation allows for an efficient analysis and easy interpretation of the LD patterns across the genomic regions studied.

Table I. Baseline characteristics of patients with schizophrenia and controls.

A, Characteristic (sex)		
Subjects	Male	Female
All subjects (n=400)	206 (51.5%)	194 (48.5%)
Patients (n=200)	164 (82%)	36 (18%)
Healthy controls (n=200)	42 (21%)	158 (79%)
B, Characteristic (mean age, years)		
Patients	42.09±58	
Healthy controls	57.38±73	

Results

Demographic characteristics of the sample population. A summary of the demographic data of the patients with SZ and controls is presented in Table I. The average age of the control group was ~57 years (range, 18-76 years), while it was 42 years in the SZ group (range, 19-78 years). The case and control groups exhibited a significant difference in their mean age. Among the SZ group, there were 36 female and 164 male patients and in the control group, there were 158 female and 42 male healthy individuals.

Identification of VD metabolic pathway gene SNPs. T-ARMS-PCR was carried out to amplify the DNA fragments of the eight SNPs in VD metabolic pathway genes, including *CYP2R1* (rs10741657), *CYP27B1* (rs10877012 and rs4646536), *CYP24A1* (rs6013897) and *VDR* (rs2228570, rs1544410, rs731236 and rs7975232). T-ARMS-PCR is a genotyping method designed to detect SNPs by utilizing two pairs of primers in a single PCR reaction: One pair flanking the SNP (outer primers) and another pair that specifically anneals depending on the allele presence (allele-specific or inner primers). This technique generates two products per allele: One common product and one allele-specific product, which allows for the determination of the zygosity of the sample directly by gel electrophoresis (36). While T-ARMS-PCR is efficient for

Table II. Genotype frequencies of the studied SNPs in the sample population.

Gene	SNP	Genotype	Healthy controls n=170 (%)	Patients n=200 (%)	OR (95% CI)	P-value
CYP2R1	rs10741657	GG	134 (79)	112 (56)	1	<0.0001
		AG	30 (18)	72 (36)	2.87 (1.75-4.71)	
		AA	6 (4)	16 (8)	3.19 (1.21-8.43)	
CYP27B1	rs10877012	GG	152 (89)	144 (72)	1	<0.0001
		GT	18 (11)	50 (25)	2.93 (1.63-5.26)	
		TT	0 (0)	6 (3)	-	
	rs4646536	AA	104 (61)	140 (70)	1	0.18
		AG	58 (34)	51 (26)	0.65 (0.41-1.03)	
		GG	8 (5)	9 (4)	0.84 (0.31-2.24)	
CYP24A1	rs6013897	TT	72 (42)	129 (64)	1	<0.0001
		TA	96 (56)	47 (24)	0.27 (0.17-0.43)	
		AA	2 (1)	24 (12)	6.70 (1.54-29.16)	
VDR	rs2228570	AA	7 (4)	10 (5)	1	0.81
		AG	78 (46)	96 (48)	1.11 (0.73-1.69)	
		GG	85 (50)	94 (47)	1.29 (0.47-3.54)	
	rs1544410	CC	47 (28)	63 (32)	1	0.45
		CT	86 (51)	88 (44)	0.76 (0.47-1.23)	
		TT	37 (22)	49 (24)	0.99 (0.56-1.75)	
	rs731236	AA	63 (37)	75 (38)	1	0.48
		AG	82 (48)	87 (44)	0.89 (0.57-1.40)	
		GG	25 (15)	38 (19)	1.28 (0.70-2.34)	
	rs7975232	CC	27 (16)	19 (10)	1	0.15
		CA	75 (44)	101 (50)	1.14 (0.74-1.78)	
		AA	68 (40)	80 (40)	0.60 (0.31-1.17)	

P-values were calculated using χ^2 test or Fisher's exact test. A Fisher's exact test was employed for rs10877012 and rs6013897, as these SNPs exhibited expected cell counts <5 in >20% of the cells. SNPs, single nucleotide polymorphisms; OR, odds ratio; CI, confidence interval VDR, vitamin D receptor.

detecting known variants, Sanger sequencing is essential for validation. Due to budget constraints, the cost of sequencing and limited local sequencing facilities, Sanger sequencing was not possible for utilization in the present study. Therefore, the T-ARMS-PCR method was utilized as a practical alternative for variant detection. Figs. S1-S8 illustrate the T-ARMS-PCR genotyping results for the studied SNPs.

Genotype and allele frequencies of SNPs among patients and controls

CYP2R1 (rs10741657; A>G). The genotype and allele frequencies of rs10741657 among all participants are summarized in Tables II and SII, respectively. Statistical analysis of the results revealed a significant difference in genotype and allele frequencies among patients and controls (P<0.0001). The findings also suggested that the AA genotype and the A allele were more prevalent among patients with SZ compared with the controls.

CYP27B1 (rs10877012; G>T). The distribution of rs10877012 genotypes and alleles among patients with SZ and controls are shown in Tables II and SII. A significant difference was shown in both genotype and allele frequencies between the two groups (P<0.0001). The results also indicated a higher prevalence of the TT genotype and the T allele in patients with SZ compared with the controls.

CYP27B1 (rs4646536; A>G). The genotype and allele frequencies among patients with SZ and controls are revealed in Tables II and SII. Statistical analysis of the data revealed no significant differences in both genotype and allele frequencies between the two groups (P=0.18 and 0.165, respectively).

CYP24A1 (rs6013897; T>A). The genotype and allele distributions of the rs6013897 SNP in patients with SZ and controls are shown in Tables II and SII. The AA genotype was significantly more frequent in the patient group (P<0.0001). Regarding the allele frequency, there was no statistically significant association identified; however, the A allele appeared to be slightly more frequent in the patient group.

VDR (rs2228570; A>G). The frequencies of the rs2228570 genotypes and alleles among patients with SZ and controls are revealed in Tables II and SII. The genotype and allele distributions were not significantly different between patients and controls (P=0.81 and 0.26, respectively).

VDR (rs1544410; C>T). The genotype and allele frequencies for the rs1544410 SNP are presented in Tables II and SII. Data analysis revealed no significant differences between patients and controls regarding both genotype and allele distributions (P=0.45 and 0.88, respectively).

Table III. Significance of differences in frequency of assigned SNPs among males and females of both study groups.

	Control		Patients	
Gene/SNP	χ^2	P-value	χ^2	P-value
CYP2R1 (rs10741657)	2.92	0.57	1.48	0.48
CYP27B1 rs10877012)	1.20	0.55	1.90	0.39
*CYP24A*1 (rs6013897)	0.69	0.95	0.45	0.80

χ^2 test or Fisher's exact test was used to analyze the significance of differences in frequency of assigned SNPs. SNPs, single nucleotide polymorphisms.

VDR (rs731236; A>G). The frequencies of genotypes and alleles for rs731236 among the two groups are summarized in Tables II and SII. Data analysis revealed no significant differences between patients and controls in both genotype and allele distributions (P=0.48 and 0.51, respectively).

VDR (rs7975232; C>A). The genotype and allele frequencies of the rs7975232 SNP are illustrated in Tables II and SII. Statistical analysis of genotype and allele frequencies revealed no significant difference between patients with SZ and controls (P=0.15 and 0.21, respectively).

Based on the findings obtained, three SNPs: *CYP2R1* (rs10741657; A>G), *CYP27B1* (rs10877012; G>T) and *CYP24A1* (rs6013897; T>A) exhibited statistically significant differences between the two groups. To evaluate the impact of sex variation on the results, a Pearson's χ^2 test or Fisher's exact test was conducted, which revealed no significant differences in the frequency of SNPs among controls (male and female controls): χ^2=0.685, P=0.953 for *CYP24A1* (rs6013897; T>A); χ^2=1.20, P=0.549 for *CYP27B1* (rs10877012; G>T); and χ^2=2.92, P=0.571 for *CYP2R1* (rs10741657; A>G). Similarly, there were no significant differences in the frequency of these SNPs among patients (male and female patients): χ^2=0.449 (P=0.799) for *CYP24A1* (rs6013897; T>A); χ^2=1.90 (P=0.387) for *CYP27B1* (rs10877012; G>T); and χ^2=1.48 (P=0.477) for *CYP2R1* (rs10741657; A>G) (Table III). These results confirmed that male or female sex does not have a significant impact on the genotype frequency between patients and controls.

HWE. Differences between observed and expected genotype frequencies for each SNP were determined by χ^2 test or Fisher's exact test to assess the deviation from HWE and to identify any possible genotyping error that could exist. As demonstrated in Table IV, the genotype and allele frequency of five SNPs (rs10741657, rs10877012, rs1544410, rs731236 and rs7975232) were in HWE. However, two SNPs (rs6013897 and rs2228570) deviated significantly from HWE in both cases and controls.

VD is decreased in patients with SZ. The Mann-Whitney U test was utilized to compare VD levels between individuals with SZ and controls, as shown in Fig. 1. The results demonstrated that VD levels were significantly lower (P<0.0001) in patients with SZ (13.8 ng/ml) compared with those in the control group (31.3 ng/ml), indicating that the levels of VD varied significantly between the two groups.

rs10741657 has the highest discriminative capacity. The area under the curve (AUC) analysis was conducted to assess the predictive performance of genetic variants in distinguishing between patients with SZ and controls (Fig. 2). The AUC values for each test result variable (rs2228570, rs1544410, rs731236, rs7975232, rs6013897, rs10877012, rs4646536 and rs10741657) ranged from 0.422 to 0.615. Notably, rs10741657 exhibited the highest AUC value of 0.615, indicating improved discriminative ability compared with the other variants, whereas rs6013897 showed the lowest AUC value of 0.422. The statistical significance of the AUC values varied across the tested variants, with rs10877012 and rs10741657 demonstrating significant discriminative abilities (P=0.003 and P<0.001, respectively). These findings suggested varying levels of predictive power among the tested genetic variants in distinguishing schizophrenic states, with rs10741657 revealing the most promising discriminatory performance.

SZ predictor SNPs. The binary logistic model revealed significant associations between genetic variants, VD levels and the likelihood of having SZ, as shown in Table V. Notably, rs10741657, rs10877012 and rs6013897 exhibited an increased likelihood of developing SZ. For rs10741657, the AA genotype had an OR of 4.911, although this was not significant (P=0.093), while the TA genotype had an OR of 2.497 (P=0.022). Regarding rs10877012, the GT genotype had an OR of 2.369 (P=0.087), however this was not statistically significant. For rs6013897, the AA genotype had an OR of 13.087 (P=0.096) without statistical power, while the TA genotype had an OR of 0.267 (P<0.001) indicating a protective effect and suggesting that having the variant decreased the probability of having SZ. Additionally, increased VD levels were revealed to be associated with a lower probability of developing SZ, with an OR of 0.888 (P<0.001). Finally, the overall model exhibited a strong statistical significance (χ^2=77.209, P<0.001) and good fit (Hosmer and Lemeshow goodness-of-fit test: χ^2=4.451, P=0.814), underscoring the robustness of the associations identified (data not shown).

LD analysis. LD analysis between all eight SNPs is shown in Fig. S9. The LD heatmap revealed that these SNPs do not exhibit significant associations, suggesting an independent inheritance within the study population. Notably, no SNP pairs demonstrated high LD (R^2 values close to 1.0), which would indicate a tendency to be co-inherited.

Table IV. Hardy-Weinberg equilibrium tests for SNPs in the case and control groups.

		Controls			Patients		
Gene/SNP	Genotype	Observed genotype	Expected genotype	P-value	Observed genotype	Expected genotype	P-value
CYP2R1 (rs10741657)	A/A	6	3.2	0.08	16	13.5	0.36
	A/G	37	42.5		72	77	
	G/G	143	140.2		112	109.5	
CYP27B1 (rs10877012)	G/G	175	175.4	0.50	144	142.8	0.52
	G/T	18	17.2		50	52.4	
	T/T	0	0.4		6	4.8	
CYP27B1 (rs4646536)	A/A	114	114.3	0.91	140	137	0.13
	A/G	62	61.5		51	57.1	
	G/G	8	8.3		9	5.95	
CYP24A1 (rs6013897)	T/T	76	90.4	<0.001	129	116.3	<0.001
	T/A	106	77.1		47	72.4	
	A/A	2	16.4		24	11.3	
VDR (rs2228570)	A/A	7	13	0.03	10	16.8	0.02
	A/G	88	76		96	82.4	
	G/G	105	111		94	100.8	
VDR (rs1544410)	C/C	57	56.2	0.82	63	57.2	0.10
	C/T	98	99.6		88	99.5	
	T/T	45	44.2		49	43.2	
VDR (rs731236)	A/A	75	75.6	0.85	75	70.2	0.16
	A/G	96	94.7		87	96.6	
	G/G	29	29.6		38	33.2	
VDR (rs7975232)	C/C	33	30.4	0.43	19	24.2	0.11
	C/A	90	95.2		101	90.7	
	A/A	77	74.4		80	85.2	

Differences between observed and expected genotype frequencies for each SNP were determined by χ^2 test or Fisher's exact test. SNPs, single nucleotide polymorphisms; VDR, vitamin D receptor.

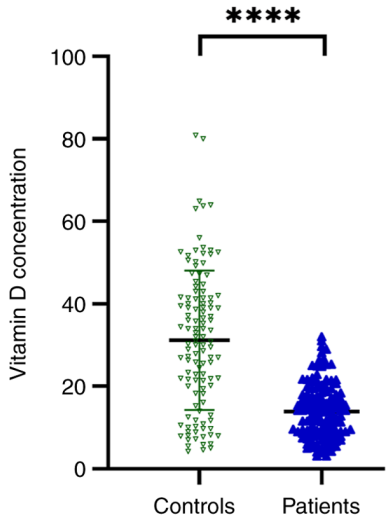

Figure 1. Comparison of vitamin D concentration between the patients with schizophrenia and the control group using Mann-Whitney U test. The mean serum VD levels were 13.8 and 31.3 ng/ml for patients with SZ and controls, respectively. ****P<0.0001.

Given the lack of significant LD, the haplotype construction from these SNPs would likely result in arbitrary combinations of alleles, as these do not represent true biological interactions. This undermines the utility of haplotype analysis in this context, as all SNPs do not appear to influence the phenotype collectively. Instead, each SNP contributes independently, which aligns with the observed distribution of allele frequencies and LD patterns among the studied SNPs.

Discussion

SZ is considered one of the most severe and complex psychiatric disorders with a strong hereditary tendency. Accumulating studies have suggested that SZ is potentially linked to disruptions in brain development that are induced by the gene-environment interplay (12,37-39). However, investigations into the factors and the underlying pathophysiological mechanisms of this disease remain a concern for researchers. SNPs have increasingly become the most popular genotyping approach in association studies due to their genetic stability and high abundance in the genome (9,40,41).

Table V. Logistic regression analysis of significant single nucleotide polymorphisms and vitamin D levels.

Variables	B	S.E.	Sig.	Exp (B)	95% CI for Exp (B)
CYP2R1 (rs10741657) A/A	1.591	0.9478	0.093	4.911	0.968-42.925
CYP2R1 (rs10741657) A/G	0.915	0.4008	0.022	2.497	1.155-5.599
CYP27B1 (rs10877012) G/T	0.863	0.5044	0.087	2.369	0.910-6.673
CYP24A1 (rs6013897) A/A	2.572	1.5457	0.096	13.087	1.147-457.792
CYP24A1 (rs6013897) T/A	-1.319	0.3586	<0.001	0.267	0.130-0.534
Vitamin D	-0.118	0.0174	<0.001	0.888	0.856-0.917

CI, confidence interval.

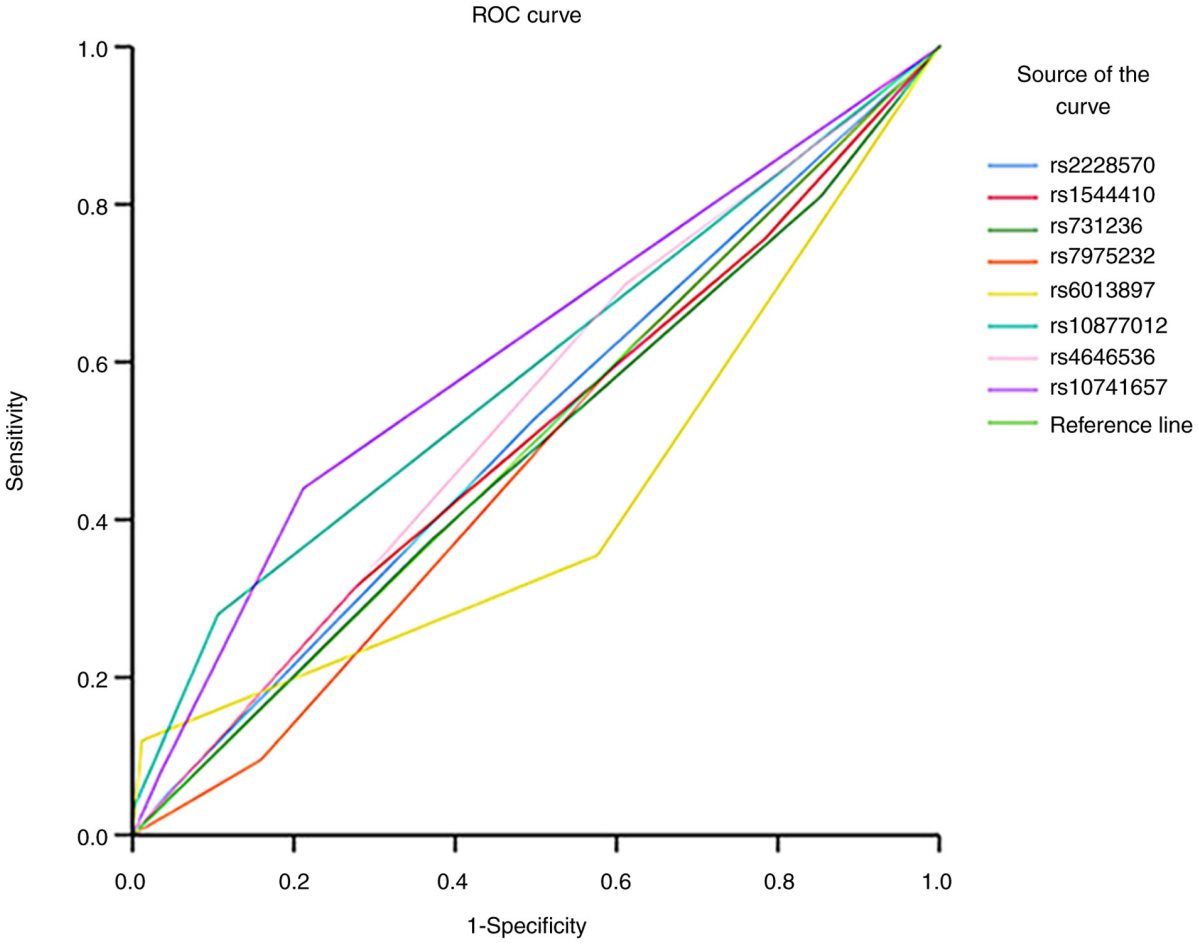

Figure 2. ROC analysis of the studied single nucleotide polymorphisms. ROC, receiver operating characteristic.

Several studies have revealed a strong relationship between VD and the pathological mechanisms of SZ (22,42-44). Some common SNPs of the VD metabolic pathway genes have been revealed to be associated with the levels of circulating VD in several diseases. The present study was conducted to investigate the association of selected SNPs in VD metabolic genes with SZ in the Jordanian population. Notably, three of these genes are involved in VD synthesis (*CYP2R1*, *CYP27B1* and *CYP24A1*), while the fourth gene contributes to its *VDR* (25,32,45,46). The studied SNPs were selected due to their potential role as key regulators in the VD pathway and their possible influence on the gene/enzyme expression and function, which are associated with altered VD serum levels. The genotype and allele frequency of the SNPs, and their distribution between the SZ and control groups were examined. Although to the best of the authors' knowledge, the associations of *CYP2R1* (rs10741657), *CYP27B1* (rs10877012 and rs4646536), *CYP24A1* (rs6013897) and *VDR* (rs2228570, rs1544410, rs731236 and rs7975232) with SZ have not been reported in case-control studies before, the present study demonstrated associations of *CYP2R1* (rs10741657), *CYP27B1* (rs10877012) and *CYP24A1* (rs6013897) SNPs with SZ among Jordanians. Such associations have not been established before in any other population.

CYP2R1 is one of the key genes that is involved in VD metabolism. It encodes a 25-hydroxylase, an enzyme

responsible for converting inactive pre-VD to 25(OH)D in the liver (47). In the present study, no deviation from the HWE for *CYP2R1* (rs10741657; A>G) was observed. It was revealed that the A allele and AA genotype of this SNP were more frequent in SZ cases (P<0.0001) compared with controls. Based on this result, the A allele may be significantly associated with SZ susceptibility, while the G allele could confer protection. Thus, the findings of the present study reveal a novel association of this SNP with SZ. Consistent with these findings, Wang *et al* (48) detected a higher frequency of the A allele and AA genotype in the Chinese Han population, and their association with an increased risk of coronary heart disease. Conversely, a study in the German population revealed a significant association between GG and GA genotypes and type 1 diabetes mellitus, suggesting the G allele as a risk allele (49). Differences between these findings are primarily attributed to the ethnic backgrounds of the studied populations and differences in sample sizes. To date, to the best of the authors' knowledge, there is no study that has evaluated the effect of *CYP2R1* (rs10741657) on the progression of SZ or any other mental disorder. As for *CYP27B1*, it is another key gene in VD metabolism required for the hydroxylation of 25(OH) D in the kidney to produce [1,25(OH)2D] (50). In the present study, no significant difference was detected between patients with SZ and controls for *CYP27B1* (rs4646536). However, a statistically significant difference was revealed in genotype and allele frequencies for *CYP27B1* (rs10877012) between patient and controls groups (P<0.0001). It was revealed that both the TT genotype and T allele of rs10877012 were more frequent in patients with SZ compared with the controls (P<0.0001), suggesting that the T allele may confer a risk role in SZ susceptibility. To the best of the authors' knowledge, no studies have examined the association between these two SNPs and SZ or any other mental disorder in any population. The *CYP24A1* gene is another gene in the VD metabolic pathway, which encodes a degradative enzyme that regulates circulating VD (51). For this gene SNP (rs6013897), a significant difference was revealed in genotype distribution between SZ cases and controls (P<0.0001). The results revealed that the TT genotype was more frequent by 8-fold in patients with SZ compared with the controls (P<0.0001). Regarding the allele frequency, the T allele frequency was slightly higher in patients compared with the controls, however it was not statistically significant. This finding requires more samples to be analyzed. This SNP has not previously been reported to be associated with any psychiatric diseases. Notably, the genotype frequencies of *CYP24A1* (rs6013897) deviated from the HWE. This deviation could be due to several reasons including: i) The small sample size, ii) the high consanguinity rate (non-random mating) in the Jordanian population, iii) genotyping error, iv) copy number variation, v) population substructure and vii) migration of individuals (52-54). Therefore, the analysis should be repeated on a larger sample size and genotyping should be carried out using different techniques such as direct sequencing or TaqMan probe assay.

The last gene assessed in the present study was *VDR*, which encodes a nuclear hormone receptor that is expressed in several tissues and cells (55). The action of VD is mediated through its binding with VDR. Polymorphisms of the *VDR* gene have been reported and evaluated as genetic risk factors in various disorders, such as issues with cognitive functioning and depressive symptoms in old age (56), Alzheimer's disease (57), mild cognitive impairments and autism (58). In the present study, four common SNPs of the *VDR* gene (rs2228570, rs1544410, rs731236 and rs7975232) were examined. The genotype and allele frequencies revealed no significant difference between the SZ and control groups. The findings of the present study align with a previous study conducted by Yan *et al* (32), who investigated the *VDR* gene variant frequencies among 100 individuals with SZ and 189 control subjects. This study also revealed a lack of associations between these SNPs and SZ. Moreover, the results of Handoko *et al* (33) support these findings, confirming the absence of an association between *VDR* gene variants and SZ.

Finally, the results of the present study demonstrated significant differences in VD levels across the two groups. The VD serum levels were revealed to be significantly lower in the SZ group compared with those in the control group with mean serum VD levels of 13.8 and 31.3 ng/ml for patients with SZ and controls, respectively (Fig. 1). Similarly, previous studies have supported the results of the present study, indicating the high prevalence of VD deficiency among patients with mental disorders, specifically SZ (42,59-62).

One of the primary limitations of the present study is the insufficient sample size, which may have prevented the detection of certain associations. In addition, the potential clinical subtypes of SZ based on symptom profiles were unable to be investigated, even though it is widely recognized that SZ has a broad spectrum of symptoms. The present study did not focus on the clinical description of the patients, since a number of them had received medication for several years, while others started taking medication at the time of sample collection and others were not on regular medication. Therefore, the present study focused on a broader diagnosis of SZ, rather than subclassifying it into specific clinical subtypes to establish foundational SNP associations. Furthermore, the genotyping and VD measurement were not performed in the same samples. Hence, it was not possible to further analyze the association between gene polymorphisms and VD status. Moreover, a potential bias was introduced by the unequal sex ratios in the sample population, which could influence the generalizability of the findings of the present study. Ensuring sex-matched samples in future studies is important to avoid discrepancies that could affect the results, particularly in genomic studies where biological sex may influence genetic expression, methylation pattern and disease outcomes. The omission of sex from the logistic regression analysis was justified by the lack of association between sex and SNPs; however, more balanced sex representation remains an important consideration for future research.

In conclusion, the present study is an association study that highlights the possible association between SNPs and SZ. Moreover, known polymorphisms with high allele frequencies in the general population are presented including rs10741657 (AF, 0.7344) and rs10877012 (AF, 0.6501), similarly to other SNPs mentioned in the present study. Finally, the importance of experimental validation through functional studies is recognized. While the study primarily focuses on genetic association analysis, the authors are committed to exploring opportunities for future investigations to directly address the limitations of the present study.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

MS conceptualized, supervised and provided administrative support for the present study, in addition to writing, editing and reviewing the original draft, designing the methodology, and analyzing and validating the raw data. RD performed the experiments, wrote the original draft and collected the samples. AAZ analyzed and curated the raw data and wrote the original draft. AGK performed clinical assessment. ABD performed the experiments and collected the samples. MS and AAZ confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participates

Ethical approval was obtained from the Institutional Review Board Committee (approval no. 2019/626) of the Jordan University of Science and Technology. Written informed consent was obtained from all participants or next of kin before enrollment in the present study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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