Genetic Susceptibility of Vitamin D Receptor Gene Polymorphisms on Autosomal Recessive Primary Microcephaly Patients in Pakistani Population: A Case-Control and In-Silico Study

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Abstract

Background

Autosomal recessive primary microcephaly (MCPH) is a rare genetic disorder that leads to reduced cerebral cortex caused by a mutation in corticogenesis. The expression of the *Vitamin D receptor (VDR)* gene is involved in the proliferation and differentiation of neural stem cells, and *VDR* polymorphisms have been associated with various neurological disorders. However, their relationship with MCPH has not been explored. This study aimed to investigate the association of *VDR* polymorphisms with MCPH and its *In-silico* analysis.

Methods and Results

Blood samples of 64 MCPH patients and 52 controls were collected to genotype *VDR* SNPs (TaqI (rs731236), FokI (rs2228570) and BsmI (rs1544410)) . *In-silico* tools were also used to assess the effects of exonic SNPs on mRNA and protein structure and pathogenicity of exonic and intronic SNPs. The study found that serum 25-OH vitamin D3 levels were significantly different in MCPH patients and healthy controls (P=0.000). The genetic analysis showed that *VDR* polymorphisms of FokI and BsmI were seven times more frequent in MCPH patients than in controls (P<0.05) and the dominant model for TaqI and recessive model for BsmI polymorphisms were also associated with the pathogenesis of MCPH. *In-silico* analysis showed that the pathogenicity effects of rs2228570 and rs1544410 are neutral while rs731236 causes a silent mutation which has no effect on VDR protein.

Conclusion

VDR polymorphisms of FokI and BsmI are associated with the risk of MCPH. These findings suggest that VDR polymorphisms play a role in MCPH, which could provide important insights for understanding the molecular mechanisms of the disease.

Introduction

Autosomal recessive primary microcephaly (MCPH) is a neurodevelopment condition in which the affected born with less than 3 standard deviation (SD) smaller head circumference (HC) than expected compared to other infants of the same gestational age, sex, and ethnic background [1]. MCPH is a complex neurodevelopmental condition and associated with mild to moderate mental retardation and no obvious structural abnormalities in the brain [2]. The rate of occurrence of primary microcephaly is about 1 in 10,000 in consanguineous population e.g. Pakistan, whereas it is estimated around 1 in 250,000 in non-consanguineous populations [3]. There are thirty genes (MCPH1-30) (*MIM: PS251200*) and different environmental factors involved in the pathogenesis of MCPH [3].

1,25-Dihydroxyvitamin D3 (1,25(OH)2D3) is recognized as a central nervous system neurosteroid and it is significant for its role in neuroprotection, neurodevelopmental and neurogenesis [4]. 1,25(OH)2D3-binding *vitamin D receptor (VDR)* is an intracellular hormone receptor that mediates the actions of vitamin D and the existence of the *VDR* in the brains of animal models and humans was reported [5]. The expression of *VDR* plays a potential role for vitamin D in neural stem cell proliferation and differentiation [4]. *VDR* gene is mapped to chromosome 12q12-q14 with 14 exons [6]. *VDR* gene is around 75 kb long and encodes a protein with 48.3 kD of molecular mass and comprised on 427 amino acids [7].

Several VDR gene polymorphisms have been identified in *VDR* gene for the susceptibility of neurodegenerative disorders including TaqI, FokI, BsmI, ApaI and Cdx2 [8]. In neurological disorders three prevalent single nucleotide polymorphisms (SNPs) of the VDR gene namely TaqI, FokI and BsmI have found to be significantly associated [9]. TaqI is located at exon 9 and due to this polymorphism the binding specificity of vitamin D is altered results from a change in protein structure of VDR [10]. TaqI (rs731236) polymorphism was significantly associated with autism disease confirmed in Polish population [11]. Turkish population and Iranian populations were not associated with TaqI (rs731236) for Parkinson disease [12, 13]. FokI is positioned at the start codon of exon 2 and intronic site is altered in the result of this polymorphism which is ultimately generate two proteins of different size [10]. Parkinson disease in Italian population and Chinese population was found to be associated with FokI (rs2228570) polymorphism [14, 15]. FokI (rs2228570) polymorphism with temporal lobe epilepsy was also studied in

Chinese population and revealed its neuroprotective roles [16]. BsmI is located at intron 8 which affects gene expression through the regulation of mRNA stability [17]. BsmI (rs1544410) polymorphism was also significantly associated in Alzheimer disease in Silesian Population [18].

MCPH is a neurodevelomental disorder and the association of *VDR* polymorphism with MCPH is not reported yet worldwide. Therefore, the aim of the present study was to investigate possible correlations between the VDR gene polymorphisms, specifically in TaqI (rs731236), FokI (rs2228570) and BsmI (rs1544410) and the Primary microcephaly in Pakistani population and to elucidate the impact of these SNPs on the structure and function of VDR along with their pathogenic effects.

Materials & Methods

The study was started after taking approval from Ethical Review Board (ERB) of Department of Biotechnology of Lahore College for Women, University Lahore. After the approval of the study, 116 participants were recruited including 52 unrelated healthy controls and 64 patients who were affected with autosomal recessive primary microcephaly according to the Declaration of Helsinki.

After the identification of participants, detailed consent was obtained. As the affected individual with MCPH are unable to communicate and understand due to intellectual disability so their parents or guardians signed the consent form for the study whereas control group signed for themselves. The inclusion criteria for the enrolment of the patients was OFC at birth <-3 S.D below the mean for gender, age and ethnicity, and various degrees of mental retardation whereas the inclusion criteria for unrelated healthy controls was the absence of all the associated features such as seizures, epilepsy, short stature, awkward gait, hearing problems, diabetes mellitus, pigmented skin, bone issues and eye disorders with MCPH.

Blood sampling and DNA isolation

The blood samples were collected with a sterile syringe by puncturing the vein in purple capped disodium ethylene diamine tetra acetic acid (EDTA) containing tube for genotype analysis and yellow capped gel and clot activator tube to collect serum for biochemical analysis. The DNA was isolated using phenol-chloroform method (organic method) [19]. Extracted DNA was quantified using NanoDrop (ThermoFisher Scientific, NanoDrop™ One/OneC, California) and stored at -20^{0}C.

Genotyping for VDR polymorphism using RFLP

Three SNPs namely TaqI (rs731236), BsmI (rs1544410) and FokI (rs2228570) in *VDR* has been selected for their potential role and their position in *VDR* gene. The genotyping was performed using RFLP PCR analysis (Applied Biosystem, Germany). The primers for two SNPs (TaqI andBsmI) were already published in literature [20] whereas the primers (Forward: 5'-GATGCCAGCTGGCCCTGGCACTG, Reverse: 5'-ATGGAAACACCTTGCTTCTTCTCCCTC) for FokI was designed through Primer 3 Plus tool (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and validated through UCSC human genome browser (https://genome.ucsc.edu/cgi-bin/hgPcr). The PCR reaction was comprised on 25µl of ready to use Green PCR master mix (Thermoscientific Dream TaqTM), 2µl of forward primer, 2µl of reverse primer, 19µl of nuclease free water and 2µl of DNA template. The PCR consisted of a hot start at 95°C for 3 min followed by 35 cycles of 95°C for 30 s, 55–65°C for 30s and 72°C for 60s. Amplified fragments were digested with the TaqI (Cat. ER0671), FastDigest FokI (Cat. FD2144) and Mva1269I (BsmI) (Cat. ER0671) restriction enzyme (Thermo Fisher Scientific) according to the manufacturer's instructions and visualized on a 2% agarose gel (Fig. 1 (a, b, c).

Statistical Analysis

The statistical analysis was performed using SPSS statistical software (version 20.0). Descriptive statistics was used to analyze the frequency, percentage, mean and SD. The genotyping frequencies of *VDR* gene polymorphism in patients with MCPH and controls was determined using Hardy-Weinberg equillibrium (HWE). Binary logistic regression was used to analyzed

the odd ratios (OR) and 95% confidence intervals (CI) to compare both allele frequencies in MCPH patients and control groups. The risk of association of MCPH with vitamin D was estimated via wild-type genotype vs. Wild/mutant (heterozygous) and mutant type (homozygous) genotypes with ≤ 0.05 p-value considered statistically significant.

In-silico Analysis

An in silico analysis was performed to evaluate the potential biological impacts of *VDR* Single Nucleotide Polymorphisms (TaqI (rs731236) and FokI (rs2228570) in their exonic regions. Furthermore, BsmI (rs1544410), located within Intron 8 of VDR gene, was also assessed through in silico analysis. The nucleotide sequence for *VDR* genes (accession# NM_001364085) was extracted from the National Center for Biotechnology Information (NCBI) data bank. For subsequent applications, the coding sequence domain of *VDR* for exonic region SNPs (rs731236 and rs2228570) with both wild and mutant alleles were translated using ExPASy server (https://web.expasy.org/translate/). Mode 2 of RNAsnp (https://rth.dk/resources/rnasnp/) was used to assess the effects of exonic SNPs on *VDR* mRNA structures. Physical and chemical properties of translated sequences were assessed using ProtParam tool (https://web.expasy.org/protparam/) available through ExPASy server. To assess the effects of *VDR* SNPs (rs731236 and rs2228570) on protein secondary structure, Chou-Farman method (https://web.expasy.org/protscale/) was used, which measures relative frequencies for each amino acid within alpha helices, beta sheets, and turns. In order to determine the hydrophobic pattern of both normal and mutant proteins, the Kyte and Doolittle scale (https://web.expasy.org/protscale/) provided by ExPaSy server was used. On Kyte-Doolittle's hydropathy plot, each amino acid was given a hydrophobicity score to indicate its hydrophobic nature. To evaluate the effect of *VDR* SNPs (rs731236 and rs2228570) on protein function, SNAP (Screening for Non-Acceptable Polymorphisms) (https://rostlab.org/services/snap/) and PredictProtein (https://predictprotein.org/) web servers were used. Furthermore, three-dimensional structures for mutant and normal proteins were predicted using Phyre2 server (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) which will be utilized in other subsequent applications.

Furthermore, the conservation of VDR sequences was evaluated using an empirical Bayesian approach on the ConSurf web server (https://consurf.tau.ac.il/). As the SNP for TaqI (rs731236) was silent in nature, several I*n-silico* tools were utilized to predict functional effects of the rs2228570 SNP in human VDR. These included PolyPhen-2 (http://genetics.bwh.harvard.edu/), SIFT (http://sift.jcvi.org/), and mutation taster (https://www.mutationtaster.org/). Stability analysis of rs2228570 SNP was performed using I-Mutant adaptation 2.0 (https://folding.biofold.org/i-mutant/i-mutant2.0.html), while profile based prediction of this SNP was carried out using PhD-SNP (https://snps.biofold.org/phd-snp/phd-snp.html). Project HOPE was used to analyze the effect of the rs2228570 SNP of FokI on VDR protein. PSIPRED tool (http://bioinf.cs.ucl.ac.uk/psipred/) was employed for secondary structure prediction using specific matrices. Moreover, rs1544410 SNP is an intronic SNP and FATHMM (http://fathmm.biocompute.org.uk/) web base tool was used to analyze the functional consequences of this SNP for the non-coding variant. RegulomeDB 2.0.3 (https://regulomedb.org/) was used to identify the DNA features and regulatory elements in FokI SNP rs1544410. String database (https://string-db.org/) was utilized to examine protein protein interaction between VDR and other proteins.

Results

Demographic and Clinical Data characteristics of recruited subjects

The current study included 116 participants from different areas of Pakistan. The demographic and clinical data was collected from all the patients affected with MCPH (male (57.8%), female (42.2%)). The mean ± SD age, HC, weight and height of patients were measured significantly different from the normal healthy controls. (Table 1) The significant difference was also observed in vitamin D level in the MCPH patients and healthy controls (Table 1). The percentage of consanguinity in the data is 75.0%. The clinical parameters such as varies degree of mental retardation, cognitive delay, motor delay, seizures, absent speech, aggression and awkward gait were also recorded in recruited samples of affected individuals with MCPH (Fig. 1d).

Table 1
Anthropometric and biochemical parameter data analysis by Chi square test and descriptive statistics in MCPH patients and healthy control subjects

Parameters	MCPH Patients (Mean ± SD)	Controls (Mean ± SD)	P-Value**
Age (Years)	16.81 ± 8.940	27.54 ± 8.486	0.000
Head Circumference (S.D)	-7.25 ± 2.32	− .346 ± 0.751	0.000
Weight (Kg)	32.24 ± 13.87	56.46 ± 11.74	0.000
Height (cm)	128.38 ± 29.70	164.73 ± 11.88	0.000
25-hydroxyvitamin D (ng/ml)	20.48 ± 2.00	32.04 ± 4.48	0.000
* S.D = Standard Deviation, MR = Mental Retardation			
**Chi square test applied and P < 0.05 indicates significant difference between MCPH patients and controls.			

VDR **polymorphisms genotyping**:

The genotype frequencies of TaqI (rs731236), FokI (rs2228570) and BsmI (rs1544410) polymorphismsin *VDR* gene were studied in 64 affected individuals with MCPH and 52 controls. The data of the frequencies of TaqI alleles was in Hardy-Weinberg equilibrium (HWE) with p-value > 0.05 whereas FokI and BsmI were not consistent with Hardy Weinberg's law at the level of significance 0.05 for our study population.

The frequencies of the wild-type (CC), heterozygous (CT) and homozygous (TT) at *VDR* polymorphism site TaqI (rs731236) were 31.9%, 39.7% and 28.4% respectively and the allele frequency of TaqI C > T allele was 80.8%. For TaqI polymorphism, the CC genotype resulted to be protective against MCPH as it was present in 53.8% in normal controls whereas 14.1% of MCPH cases have CC genotype. On the contrary, the other genotype TT is said to be a risky genotype for MCPH susceptibility as the frequency of TT in MCPH is 45.3% which is highly greater than the normal controls (7.7%). in particular, the results suggest that T-allele is strongly associated with MCPH as it appeared almost 5 times more often in MCPH patients (OR 95%CI = 5.182 (2.941–9.130), P-value= (0.000)) (Table 2). In genotype analysis, there was only a significant association at dominant (CC + CT/TT) genetic model (OR (95%CI) = 9.943(3.204–30.858), P-value= (0.000)) whereas recessive and codominant model had no association with MCPH disorder (Table 3).

Table 2
Genotype and allele frequency of TaqI, FokI and BsmI polymorphism of *VDR*

	SNP		*Control(N = 52)*	*Case (N = 64)*	*OR (95%CI)*	*P-Value*
			N(%)	N(%)		
TaqI	rs731236	Genotype				
		CC	28 (53.8%)	9 (14.1%)	1	
		CT	20 (38.5%)	26 (40.6%)	0.247 (0.096–0.640)	0.004
		TT	4 (7.7%)	29 (45.3%)	0.044 (0.012–0.161)	0.000
		Allele				
		C	84 (80.8%)	44 (34.4%)	1	
		T	**20 (19.2%)**	**84 (65.6%)**	**5.182 (2.941–9.130)**	**0.000**
FokI	rs2228570	Genotype				
		CC	35 (67.3%)	33 (51.6%)	1	
		CT	14 (26.9%)	11 (17.2%)	0.833 (0.331–2.095)	0.698
		TT	**3 (5.5%)**	**20 (31.3%)**	**7.071 (1.921–26.032)**	**0.03**
		Allele				
		C	84 (80.8%)	77 (60.2%)	1	
		T	**20 (19.2%%)**	**51 (39.8%)**	**2.782 (1.523–5.082)**	**0.001**
BsmI	rs1544410	Genotype				
		GG	35 (67.3%)	29 (45.3%)	1	
		GA	13 (25.0%)	10 (15.6%)	0.928 (0.355–2.425)	0.879
		AA	**4 (7.7%)**	**25 (39.1%)**	**7.543 (2.354–24.172)**	**0.001**
		Allele				
		G	83 (79.8%)	68 (53.1%)	1	
		A	**21 (20.2%)**	**60 (46.9%)**	**3.487 (1.930–6.300)**	**0.000**

Table 3
Models proposed for TaqI, FokI and BsmI polymorphisms between MCPH cases and normal controls in Pakistani population.

SNP	Model	Genotype	Control (N = 52)	Case (N = 64)	OR (95%CI)	P-Value
			N(%)	N(%)		
rs731236	**Dominant**	**CC + CT/TT**	**24 (46.2%)**	**35 (54.7%)**	**9.943 (3.204–30.858)**	**0.000**
	Recessive	CC/CT + TT	48 (92.3%)	55 (85.9%)	0.140 (0.58 – 0.342)	0.000
	Codominant	CT/CC + TT	32 (61.5%)	38 (59.4%)	1.095 (0.518–2.315)	0.813
rs2228570	Dominant	CC + CT/TT	49 (94.2%)	44 (68.8%)	0.135 (0.037–0.484)	0.002
	Recessive	CC/CT + TT	17 (32.7%)	31 (48.4%)	1.934 (0.905–4.131)	0.088
	Codominant	CT/CC + TT	38 (73.1%)	53 (82.8%)	1.775 (0.727–4.335)	0.208
rs1544410	Dominant	GG + GA/AA	48 (92.3%)	35 (54.7%)	0.130 (0.042–0.405)	0.000
	Recessive	**GG/GA + AA**	**17 (32.7%)**	**39 (60.9%)**	**2.485 (1.162–5.314)**	**0.019**
	Codominant	GA/GG + AA	39 (75.0%)	54 (84.4%)	1.800 (0.716–4.524)	0.211

At the FokI (rs2228570) *VDR* gene polymorphism site the frequency of all three genotypes (CC,CT and TT) were observed in MCPH patients and controls. Among 116 participants, the frequency of wild-type CC was 58.6%, heterozygous CT was 21.6% and homozygous TT was 19.8%. The allele frequency of FokI C > T allele was 80.8%. The results for FokI polymorphism suggest that CC genotype was acting as protective against primary microcephaly due to its greater frequency (67.3%) in normal controls as compared to MCPH cases which has 51.5% CC genotype. On the other side, TT genotype was resulted to be risk for MCPH as it was present 31.3% in MCPH patients than normal controls only have 5.5%. moreover, the results for T-allele suggested a strong association for MCPH susceptibility as it appeared almost 2 times more in MCPH cases than normal control group (OR (95%CI) = 2.782 (1.523–5.082) P-value = 0.001)) (Table 2). In the genotype analysis, there were no significant associations at the dominant, recessive and co-dominant genetic models (Table 3).

At BsmI (rs1544410), the three GG, GA, AA genotypes were observed in both MCPH and healthy control group. Out of 116 participants, 55.2% had genotype GG, 19.8% had GA and 25.0% had AA and said to be wild-type, heterozygous and homozygous respectively. The allele frequency of BsmI G > A allele was 79.8%. In particular, the AA genotype resulted to be a risk factor for MCPH disorder as it was present in 39.1% of MCPH cases but only in 7.7% in normal control group (OR (95% CI) = 7.543 (2.354–24.172), P-value = 0.001)) whereas the other GG genotype appeared to protective against MCPH cases as it was present in 67.3% of normal control group but only in 45.3% of MCPH cases. Notably, the results suggested an association between the presence of A-allele at BsmI position and the presence of MCPH. The A allele appeared almost 3 times more in MCPH pateints (OR (95%CI) = 3.487 (1.930–6.300), P-value = 0.000) than in normal control group (Table 2). In the genotype analysis, there were a significant associations at recessive model for MCPH patients in Pakistani population (OR (95%CI) = 2.485 (1.162–5314), P-value = 0.019) whereas dominant and co-dominant genetic models were not showed association (Table 3).

In-Silico **Analysis of VDR Polymorphisms**

For In silico analysis the retrived VDR sequence was translated to get the protein sequence for other applications. ProtParam was used to determine the physical and chemical properties of VDR protein; predicted molecular formula: $C_{2091}H_{3342}N_{596}O_{648}S_{34}$ with a molecular weight: 48289.18 Da encompassing 6711atoms and an isoelectric point (pI) value of 6.08. In a normal protein, there were 60 negatively charged residues (Asp and Glu) and 53 positively charged residues (Arg and Lys). This resulted in an aliphatic index of 77.87 for this particular VDR peptide. Furthermore, the stability index for normal VDR protein was 53.26 which indicates it's unstable. The Grand Average of Hydropathicity (GRAVY) for normal VDR was calculated at -0.410 and its estimated half-life was 30 hours in mammalian reticulocytes in vitro. These parameters remain unchanged when considering at I352I phenotype as VDR rs731236 polymorphism is an A-G substitution in nucleotide 1058 of VDR, which

does not alter any amino acid sequence (p.Ile352Ile), and making this mutation silent in nature. Whereas for M1T, the physiochemical properties are summarized in Table 4.

Table 4
Physiochemical properties of *VDR* SNPs rs731236A > G and rs2228570T > C using ProtParam

Protein Phenotype	Molecular weight	Theoretical pI	Estimated half-life	Instability index	Aliphatic index	GRAVY*
Wild	48289.18	6.08	30 hours	53.26	77.87	-0.41
FokI	47927.71	6.17	7.2 hours	53.27	78.18	-0.419
*Grand average of hydropathicity						

Predicting the effects of VDR rs731236A > G and rs2228570T > C on local VDR RNA secondary structure revealed that neither SNP made any fundamental modifications to mRNA's secondary structure with p = 0.9456 and 1.000 respectively, as the p-values are greater than 0.2. Chou and Doolittle scored beta sheets at 352 for residue I, with no score for M at position 1 (rs2228570), while Kyte and Doolittle hydrophobic score for VDR at position 352 was 0.856 (Isoleucine), with no score at position 1 (Fig. 2a). Furthermore, PredictProtein revealed I352 is buried in the structure while M1 is exposed by solvent accessibility analysis; SNAPserver showed no effect of I352I substitution on protein structure (Score: -93; Expected accuracy: 97%) as well as M1T substitution on protein structure (Score: 70; Expected accuracy: 85%), which are both neutral effects (Score: -93; Expected accuracy: 97%) (Fig. 2b).

ConSurf was used for conservation analysis of M1 and I352 residues, which revealed that both amino acids are highly conserved with their conservation score being 8 (Fig. 3a). On the basis of sequence homology and physio-chemical similarity between amino acids, PolyPhen-2, SIFT, Mutation Taster and PhD-SNP were interpreted to be neutral for M1T mutation pathogenicity; I-Mutant predicted an increase in stability due to this mutation (Table 5). Project HOPE's results revealed that the mutant residue is smaller than its wild-type counterpart, is more hydrophobic, and might lead to loss of interactions. Furthermore, while the Wild Type residue remains conserved, its mutant counterpart shared some properties with it; therefore, this mutation might take place without damaging the protein (Fig. 3b). PSIPRED predicted the coils at M1 position; however, when this initiated methionine was lost the M4 act as start codons and the coil was once again at its initial position. No significant alteration in VDR structure due to M1T mutation was observed (Fig. 2c).

Table 5
In Silico Prediction of Pathogenicity

SNP ID	Poly-Phen 2		SIFT		Mutation Taster		PhD-SNP		I-Mutant	
	Effect	Score	Effect	Score	Alteration Type	Prediction	Effect	Score	Stability	RI*
rs2228570T > C	Benign	0.289	Not Tolerated	0.32	Single Base Exchange	Polymorphism	Neutral	9	Decrease	2
***Reliability Index										

FATHMM gives 0.15081 score for rs1544410 SNP of *VDR* at position chromosome 12: 48239835 which indicates that the effect of this SNP is neutral or benign as the score is below 0.5. RegulomeDB give the predicting score as 0.41198 which means this variant is most likely to be a regulatory variant and it can be predicted as transcription binding factor, any motif, DNase footprint and DNase peak due to its rank score of 2b.

String databases predict the interactions between proteins such as VDR and other proteins such as ARID1A, BAZ1B, RARG, RXRA, RXRB, RXRG, SMAD3, SMARCC1, SMARCC2 and SMARCD1 (Fig. 3c). These interactions were determined through

experiments, textmining and curation from databases, with most of these interacted proteins involved in controlling gene expression for various biological processes.

Discussion

The purpose of this research study was to investigate the association of VDR polymorphisms (TaqI, FokI and BsmI) with autosomal recessive primary microcephaly in Pakistani population. VDR polymorphisms have been potentially associated with the progression of several neurological disorders such as Parkinson, autism, Alzheimer and epilepsy disorders [8, 11, 16, 21–22].

In the current study the average mean head circumference of primary microcephaly patient is reported as -7.25 ± 2.32 S.D which is slightly below the mean that less − 3 S.D in Asian population [1]. The reduction in HC in enrolled samples is may be due to consanguinity which may leads to more frequency of severe mentally retarded cases 43.8% (28/64 cases) than mild and moderate level hence the size of skull would be more small. However, other clinical features such as cognitive and motor delay are reported e.g. 92.9% and 94.3% respectively while, epilepsy frequency (28.0%) is reported in Pakistani MCPH patients. Many studies also gives evidence of the high frequency of cognitive and motor impairment and frequent epilepsy associated with MCPH [8]. Our results are consistent with the previous reports as the lobes of cerebral cortex are fails to develop normal and play a major role in causing motor delay, aggression and difficulty in processing the information in MCPH (*MIM: PS251200*).

Biochemical analysis revealed that MCPH patients were detected with low serum 25-OH vitamin D3 (below normal 30–100 ng/ml) as compared to normal cases e.g. 20.48 ± 2.00 ng/ml and it is found to significantly different (p-value 0.000) in recruited controls and MCPH of Pakistani population. In Korean and Swedish population, hypovitamin D level has been found to be associated with Parkinson disease and ADHD, schizophrenia and autism respectively [23]. Vitamin D plays a crucial role in the development and functioning of brain. The low level of Vitamin D in serum may disturb the pathway of development of brain and ultimately the proper function of brain may altered in the result of subsequent change in the level. In Korean population, the supplementation of vitamin D helped in the prevention of deterioration of Hoehn & Yahr stage in Parkinson disease patients in clinical trials. Hence, it is suggestive to give the vitamin D supplementation to MCPH patients for the improvement in their cognitive and motor skills.

This data showed that for TaqI polymorphism (rs731236), CC genotype was protective against MCPH as the frequency of CC genotype was found to be 53.8% in normal controls and 14.1% in MCPH cases. In Turkish. Hungarian, Polish, Silesian and Iranian populations CC genotype was also revealed as a protective role against of Alzheimer's Parkinson, Autism and Alzheimer disease respectively [11–13, 21–22]. As TaqI polymorphism is involved in the alteration of binding specificity of vitamin D and ultimately play important role in the pathogenicity of neurological disorders [10].

FokI polymorphism (rs2228570) was found to be significantly associated with MCPH in Pakistani population as the results of this study showed that it was seven times more common in MCPH than in unrelated healthy controls (OR = 7.071 (95% CI: 1.921–26.032), p-value: 0.05). Turkish, Hungarian and Chinese populations have shown an association between autism, Parkinson's disease and temporal lobe epilepsy respectively [16, 21, 24]. FokI polymorphism results in the alteration of intronic site and give two proteins of different sizes in result and involves in disease progression [10]. Therefore, the study of this polymorphism is pivotal to understand its involvement in the etiology of MCPH. Expression studies on animal models will be helpful to uncover its function in MCPH.

For the association between BsmI polymorphism (rs1544410) of *VDR* gene and MCPH, GG genotype was found to be associated with neuroprotective role due to its highest frequency (67.3%) in control population whereas AA genotype was associated with risk of susceptibility of MCPH in this study as it is more than seven time increases the event of occurrence of MCPH in this case group (OR (95%CI) = 7.543 (2.354–24.172). In Turkish population AA genotype of BsmI polymorphism was also found to be associated with autism [24]. However, studies conducted in Polish and Hungarian population showed that this polymorphism was not associated with childhood autism and Parkinson disease respectively [11, 21]. Findings of Polish and Hungarian population association analysis of BsmI polymorphism and neurological disorders is not homogeneous with this

report. Gene expression studies will be helpful to understand its mechanism in MCPH pathogenicity as this polymorphism is involved in the change of stability of mRNA [17].

In order to explore the genotype phenotype association between VDR polymorphisms and MCPH, there are three genotypic test models were predicted, *i.e.* dominant, recessive, and co-dominant. The results for model prediction reveals that dominant model for TaqI polymorphism and recessive model for BsmI polymorphism were found to be associated with the 9-fold and 2-fold increase with risk of the susceptibility of MCPH disorder in this finding (OR (95%CI) = 9.943 (3.204–30.858) and (OR (95%CI) = 2.485 (31.162–5.314))with 0.000 and 0.019 p-value respectively whereas the recessive and codominant models for TaqI polymorphism and all the models for FokI and dominant and codominant models for BsmI polymorphism at *VDR* site were not associated with the disease risk. In Southeastern European Caucasian population, the dominant model of TaqI was also associated with Alzheimer's disease [22]. In another review conducted in Chinese population BsmI and FokI showed significantly association with Parkinson's disease [15]. The reason for controversial results may be the difference in ethnicity and different disease group. Moreover, more studies are suggestive to confirm these results in Asian populations specifically in Pakistan.

A novel In silico analysis for TaqI (rs731236) and FokI (rs2228570) was conducted to evaluated the effects of these SNPs on the VDR structure and expression. The results of the bioinformatics tools showed that rs731236 is silent mutation and not affecting the structure and expression of *VDR* protein as current study also showed no association for Taq (rs731236) polymorphism with MCPH. This mutation was also found to be silent in the study conducted in Polish population [11]. While rs2228570 is a missense mutation and results in the shifting of ATG hence the start Methionine is lost and resulted protein is shorter than the wild type protein [11]. This change may influence the some changes in the secondary structure of VDR but the lost is very minor and the analysis of pathogenicity prediction reveals this mutation is benign in the result of most of the In silico tools (Table 5). Bioinformatics analysis of rs1544410, an intronic SNP of VDR showed that this is the mutation of regulatory variant and it is also a benign mutation. It means the presence or absence of these variants of *VDR* gene would not lead to a significant change in the structure [11].

It is of the utmost necessity to broaden this investigation to uncover relevant variants at the genome-wide level in order to identify genetic variables associated with MCPH and comprehend its pathophysiology. In addition, the identification of genetic variations associated with MCPH has the potential to facilitate individualized carrier screening and genetic counseling for genetically at-risk people and families with MCPH.

Conclusion

This is the first report on the potential correlation between *VDR* polymorphisms and MCPH in Pakistani population. It is concluded that both FokI (rs2228570) and BsmI (rs1544410) polymorphisms of *VDR* gene are found genetically associated with primary microcephaly. While Dominant model for TaqI polymorphism (rs731236) and recessive model for BsmI polymorphism (rs1544410) are predicted to be associated for the pathogenesis of MCPH. Since serum 25-OH vitamin D3 was significantly different between MCPH and healthy controls this could indicate that the metabolism of Vitamin D might be affected when contributing to the pathogenicity of among MCPH patients.

Declarations

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Competing Interests: The authors declare that there are no conflicts of interest.

Author Contributions: Rasheeda Bashir TR-158/LCWU/1682

and Iram Anjum provided the conceptualization, Supervision, Review and Editing. Komal Aslam performed all the experimental work, optimization, validation, statistical analysis and wrote original manuscript. Kanwal Aslam performed statistical analysis of the descriptive, clinical and genotype data and review the manuscript. Rukhama Haq provided the assistance in primer selection and design for the study.

Ethical Approval: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethical Review committee of Lahore College for Women University (No. REF/NO/LCWU/BIOT/304; Dated 03-05-2021).

Consent to Participate: Written informed consent was obtained from the normal healthy controls and the parents of all affected individuals prior to the study.

Consent to Publish: The authors affirm that human research participants provided informed consent for the publication of the data presented in figure 1(d).

Data Availability: All the clinical data has been mentioned in the paper. No supplementary data is needed.

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Figures

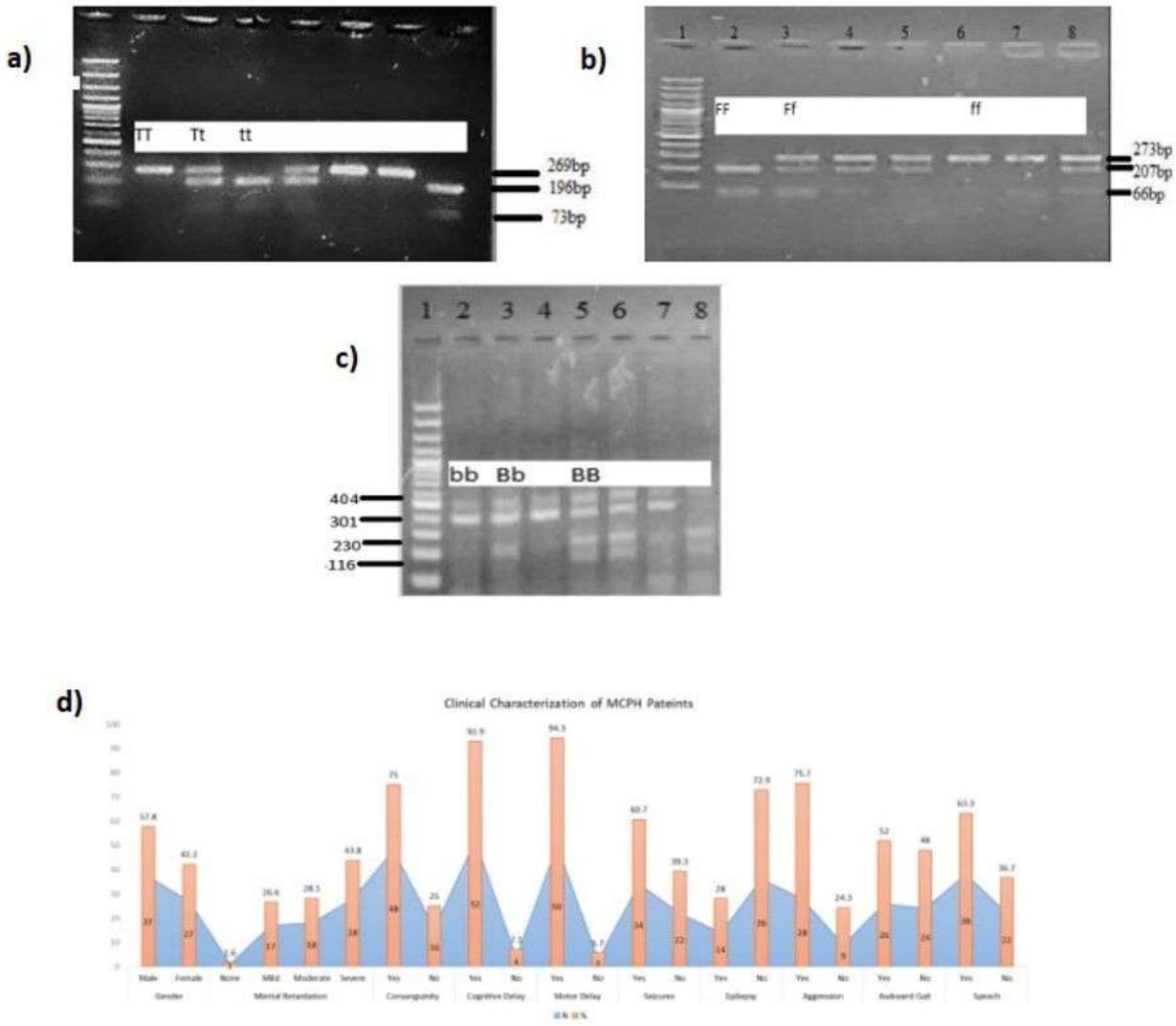

Figure 1

2% Agarose gel electrophoresis of RFLP-PCR showing wild type, heterozygous and homozygous mutant variants for (a) *VDR* SNPs TaqI enzyme, (b) *VDR* SNPs FokI enzyme, (c) *VDR* SNPs BsmI enzyme, (d) Graphical presentation of clinical data of MCPH patients

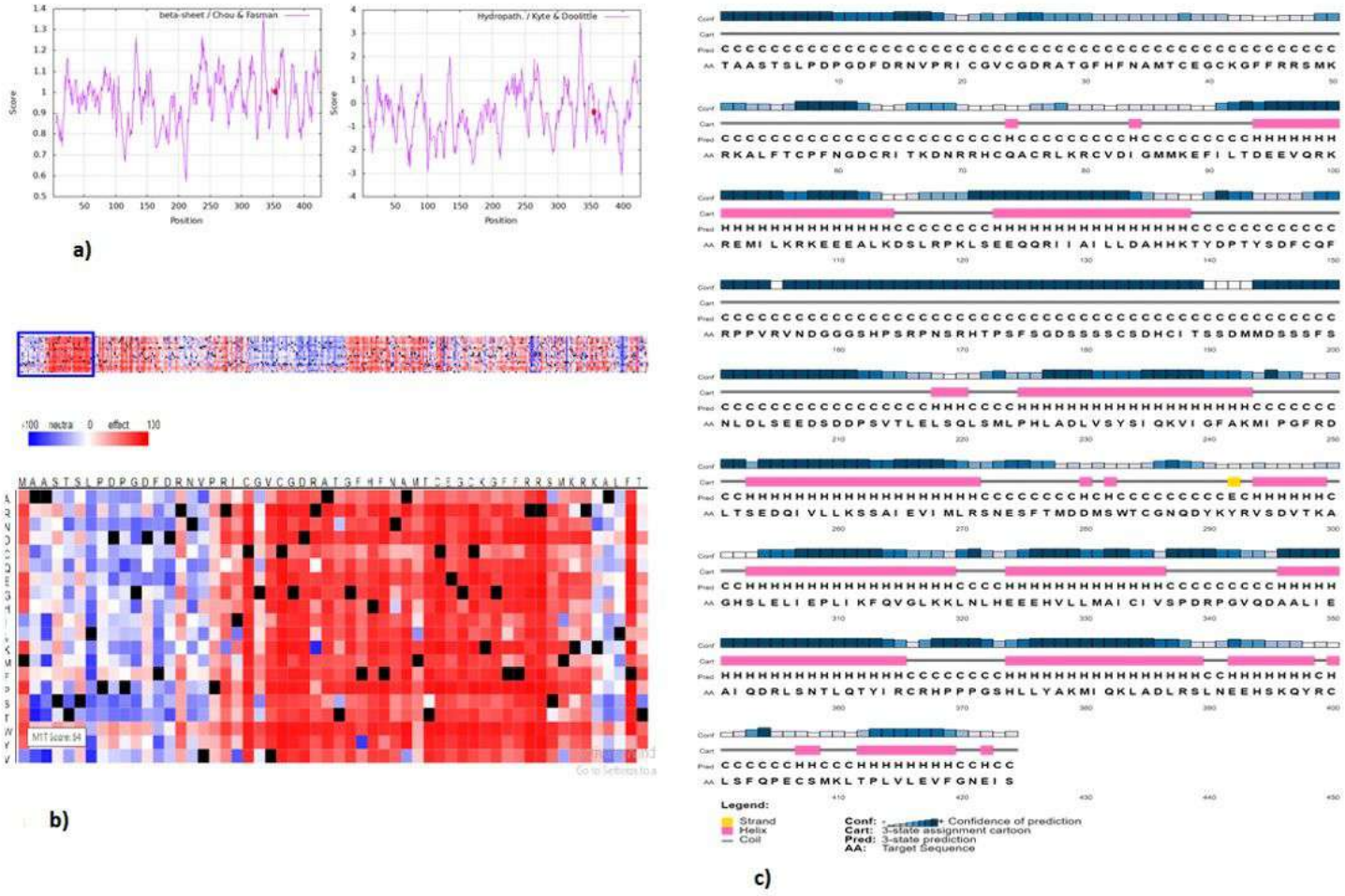

Figure 2

(a) Score for Secondary Structure Prediction by Chou and Fasman and Hydrophobicity score by Kyte and Doolittle (Red dot shows the score for 352I residue whereas M1T is not predicted by these tools), (b) The effect of M1T substitution on protein functions evaluated by SNAP Server, (c) Secondary structure of VDR for rs2228570T>C SNP predicting coils at M1T residue generated using PSIPRED with high confidence of prediction

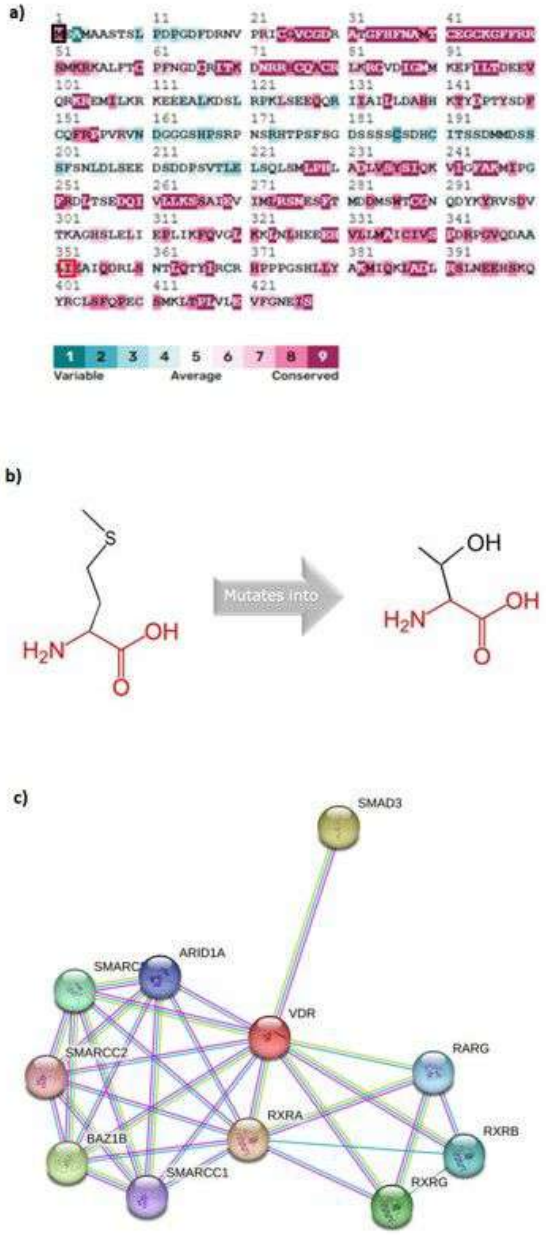

Figure 3

(a) Conservation analysis by ConSurf (black square showed the Methionine reside at position 1 with score 8 and red square showed the Isoleucine residue at position 352 with score 8, means highly conserved residues), (b) Schematic Structure for wild type Methionine (left) and mutated Threonine (right) residue for rs2228570T>C SNP of VDR generated using HOPE project, (c) Protein-Protein interaction network of VDR with other proteins generated using STRING database.