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RESEARCH ARTICLE

GENOTYPIC ANALYSIS IN BAHRAINI SICKLE CELL PATIENTS WITH HYPOVITAMINOSIS D

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ABSTRACT

Background: Patients with sickle cell disease (SCD) had a low serum level of vitamin D. Also, genotypic analysis in Bahraini SCD patient has not been investigated.
Aim: To investigate gene polymorphisms (SNP) and to observe if genotyping, allelic discrimination or allele frequency have any effects on SCD patients compared to controls.
Methods: For quantification of serum PTH, ELISA kits were used. Calcium (Ca), phosphate (Ph) and Alkaline Phosphatase (ALP) were measured using the auto analyzer (Cobas). Gene polymorphisms of VDR and PTH genes were investigated using real-time PCR.
Results: Genotypic analysis in the VDR gene revealed complete absence of homozygous allele A (the mutant allele) among both SC patients and controls. AG genotypes was more frequent than the GG genotypes in patient and controls ($p<0.012$). The percentage of allele A frequency was 61.4% and 66.1% within patient and controls, respectively with no statistical difference. Yet, the analysis showed no association within sex between genotypes and allele's frequency. In the PTH gene, the GG-genotype was more frequent than the AG genotype in patients and controls ($p<0.022$). The percentage of A allele was 34.3% in patients and 25.8% in controls, but the difference was not significant. No association was found between GG genotype in sex and allele frequency and sex in PTH gene.
Conclusion: In the VDR gene the AG genotype is associated with SCD, while in PTH gene the GG genotype is associated with SCD. There was no statistical difference in allele's frequencies between patients and controls in both VDR and PTH genes. Moreover, no association was found between VDR and PTH gene polymorphisms and their corresponding serum levels suggesting that allele A may be associated with vitamin D serum level.

INTRODUCTION

The prevalence of hypovitaminosis D has been reported to be high in various regions around the Middle East. Despite sufficient sunshine throughout the year, there are a large number of studies in the past decade suggesting that one-third of individuals living in Sub-Saharan Africa and the Middle East have deficient serum 25-hydroxyvitamin D levels (Arabi, El Rassi *et al*., 2010). The risk of vitamin D deficiency among Sickle-cell disease (SCD) almost 4 folds greater than normal(Adla, Garadah *et al.,* 2013). SCD is an autosomal recessive genetic blood disorder, characterized by red blood cell (RBC) that assumes an abnormal, rigid, sickle shape, due to a mutation that results in the substitution of the amino acid valine for glutamic acid at position six of the beta chain leading to sickle cell hemoglobin (HbS) (Ingram 1989). The sickle mutation was suggested to have arisen spontaneously at least on five occasions throughout mankind history (Bain 2006). These apparently independent mutations were identified by the presence of unique restriction sites, and analyzed by restriction fragment length polymorphisms (RFLP). There are three loci of HbS in Africa, associated with different haplotypes. The Arab/Indian (AI) or Saudi/Indian haplotype {HBB (β-globin gene) haplotype} (Alsultan, Alabdulaali *et al*., 2013) presents in eastern Saudi Arabia, Bahrain, Kuwait and Oman (Chen, Lalezari *et al*., 2005). Studies from Kuwait and Bahrain have also shown that Saudi/Indian haplotype is the most common haplotype in their SCD patients (al-Arrayed and Hamza 1995). This haplotype has been described to have a mild clinical presentation of sickle cell disease, compared to

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the other haplotypes (Powars 1991; el-Hazmi and Warsy 1996). In vitamine D receptor (VDR) gene, G allele is the ancestral allele whereas A allele is the mutant one. Mao *et al.* concludes that (A allele/AA) genotype at the BsmI site (rs1544410 or Bsml), maybe risk factors for the onset of rickets among Asians (Mao and Huang). In 1994, Morrison *et al* has established an association between the AA VDR genotype and low bone mineral density in UK population (Morrison, Qi *et al*., 1994). However, in 1996 Houston *et al.* did not find any association between low mineral density and the VDR genotype among 44 patients with severe osteoporosis with vertebral compression fractures in UK population (Houston, Grant *et al*., 1996). On the other hand, a study among 90 healthy Caucasian males demonstrated that boys with the (AA) VDR genotype were shorter at birth and grew less from birth until after puberty than their AG and GG counterparts (Lorentzon, Lorentzon *et al*., 2000). Regarding the PTH gene a SNP (rs1459015) was found to be related to serum calcium and calcium metabolism (Jorde, Svartberg *et al*., 2012). Moreover, Carling *et al.* has documented an association of calcium-mediated PTH secretion and inhibition with VDR genotype (Carling, Ridefelt *et al*., 1997). In this study the hypothesis was that there could be an association between hypovitaminosis D genotype/allele frequency and its phenotype, thus, two common SNPs were investigated, a SNP related to the VDR gene (rs1544410 or Bsml) and another SNP (rs1459015) in PTH gene were investigated in Bahraini patients with sickle cell disease and healthy controls.

MATERIALS AND METHODS

Subjects

The study was approved by the Ethics Committee of the Arabian Gulf University, Manama, Bahrain. All subjects were required to sign an informed consent form prior to entering the study. Seventy SC patients and seventy sex match controls were used in this study. For analysis of vitamin D in sera, two ml of blood was drawn from each patient in a yellow capped gel vacutainer tube in addition to 3 ml of whole blood in EDTA tube (purple capped) for further DNA and genetic analysis.

Measurement of vitamin D in sera

Serum levels of vitamin D were measured by using Ultra Performance Liquid Chromatography-interfaced with tandem Mass Spectrometry (UPLC/MS/MS) at Al Jawhara Centre for molecular medicine, The levels of Vitamin D were established according to a published study (Golbahar, Al-Saffar *et al*.). 100 µl of the serum was used for Vitamin D serum assay. The intra-assay and inter-assay coefficients of variation for determination of vitamin D in serum were 3.4% and 4.6% for low control and 3.5% and 4.4% for high control, respectively.For quantification of PTH in serum, ELISA was used (Creative Diagnostic, USA).While Calcium (Ca), phosphate (Ph) and alkaline phosphatase (ALP) were measured using the auto analyzer (Cobas).

Genotyping

For determination of genotyping of VDR and PTH gene polymorphism two SNPs were investigated, one in the VDR gene (rs1544410) and (rs1459015) in PTH gene, the assays were carried out in a total volume of 12µl, which contained 3 µl DNA template, 6 µl TaqMan genotype master mix, 1.875 µl nuclease free water and 0.3µl 40$^{\times}$ SNP primer mix, purchased from (Applied Biosystem, USA). Cycling conditions comprised: pre- and post-PCR steps. Pre-PCR (hold step) stage was performed at 60 °C for 30 sec and 95°C for 10 min (to activate the polymerase). Cycling conditions consisted of 40 cycles of denaturation (92°C for 15 sec), and combined annealing/extension (60 °C for 1.0 min), followed by a holding stage at 60°C for 30 sec

Statistical analyses

Data were entered in computer using SPSS for windows version 20.0 (SPSS Inc., Chicago, IL). Results were cross-tabulated to examine the independency between variables. Statistics was performed using χ^2-square for test of association and Fisher's exact test as appropriate. Where two continuous independent variables were examined, t-test and analysis of variance (ANOVA) were used as adequate. Logistic regression was used to explain the disease by the explanatory variables. Frequency tables were performed as descriptive statistics. A P-value of less than 0.05 was considered significant in all statistical analysis.

RESULTS

Ninety four percent (94%) of SCD patients had either deficient (64%) or insufficient (30%) levels of VD and only four patients (6%) had optimal levels (Fig1).

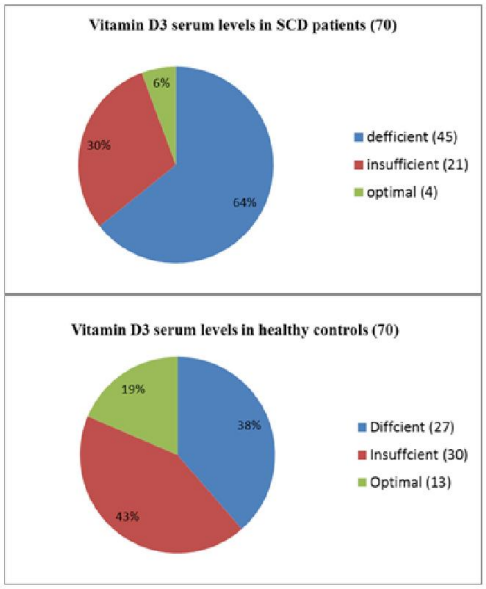

Figure 1. Vitamin D3 serum levels in SCD patients and healthy controls

On the other hand, 13 subjects (19%) of the controls have optimal levels of VD, the rest have both deficient (38%) and insufficient (43%) levels of VD (Fig. 1). Almost 84% of controls have a normal PTH levels that within the range (10-66 pg/ml), while few have lower levels (< 10 pg/ml). On the other hand, fewer SCD patients had normal PTH levels compared to

the controls and more patients had low PTH levels compared to controls, but the difference was statistically not significant. However, 10 patients had extremely high serum levels of PTH more than expected in relation to their low levels of VD and when those 10 patients were excluded the difference became statistically significant (Table 1). Furthermore, other parameters involved in VD regulation Ca, phosphate and ALP were tested for both patients and controls and the outcome was statistically significant in favor of patients (Table 2), although it showed that the Ca, phosphate and ALP levels were normal in most of patients. Results for VDR gene (depicted in Table3) showed that the frequency of AG genotypes is 84 (60%), no mutant homozygous genotype with allele A (AA) was found in VDR gene in both patients and controls. Genotype frequency of GG was 48 (34.3%).

Interestingly, allele A frequency for VDR gene is 84 (60%), which is the same result as AG genotypes due to the absence of AA homozygous state. For PTH gene results (depicted in Table 3); G allele is the ancestral allele, Genotype frequency of GG was 92 (65.7%) and genotype frequency of AG is 35 (25%), respectively. On the other hand, allele A frequency in PTH gene is 40 (28.6%). Cross tabulation (shown in Table4) carried out to examine association between genotyping and alleles frequency within sex. For VDR gene (rs1544410) AG percentage within sex is 64.6% for females and 53.4% for males, while GG within sex is 30.5% for females and 39.7 for males, no association was found (p=0.410) (Table 4). The percentage of A allele (in VDR) within sex is 67.9 % in females group and 57.4% in males group, using Chi square test no association was shown (p=0.216) (Table 5).

Table 1. Vitamin D levels and PTH in SCD patient vs controls

Patient/control		No	Mean	STD	SEM	P value	95%CI
D3	Patients	60	26.7833	15.03461	1.94096	= 0.002	-14.19 - -3.35
	controls	70	35.5586	16.03351	1.91637		
PTH	Patients	60	17.1555	17.21976	2.22306	< 0.0001	-18.23 - -6.22
	controls	70	29.3769	17.26931	2.06408		

PTH in Patient vs. Controls excluding the 10 with very high PTH serum levels

D3 = vitamin D3
PTH = parathyroid hormone

Table 2. Vitamin D levels and biochemical profile in SCD

Patient vs. control		No	Mean	STD	SEM	P-value	95%CI
D3	Patients	70	26.0829	14.4219	1.72375	<0 .0001	-14.57- -4.38
	Controls	70	35.5586	16.0335	1.9162		
Ca	Patients	70	2.2249	0.16809	0.02009	<0 .0001	.15 -.35
	Controls	70	1.9717	0.38813	0.04639		
ALP	Patients	70	122.343	59.51979	7.11398	< 0.0001	53.01-82.22
	Controls	70	54.7286	16.6637	1.9917		
Ph	Patients	70	1.1563	0.23413	0.02798	< 0.0001	-.26 --.089
	Controls	70	1.3286	0.26493	0.03166		
PTH	Patients	70	46.6409	112.13132	13.40226	NS	-9.55 -44.08
	Controls	70	29.3769	17.26931	2.06408		

D3 Vitamin D3
CaCalcium
ALPAlkaline phosphatase
PhPhosphate
PTHParathyroid hormone

Table 3. Genotype and Allele Frequency

	Frequency	Percentage
VDR* genotype		
AG	84	60
GG	48	34.3
AA	-	
Total	140	
Missing	8	
VDR* allele		
A	84	60
Others	48	34.3
PTH genotype		
AG	35	25
GG	92	65.7
AA	5	3.6
Total	140	
missing	8	
PTH allele		
A	40	28.6
**Others	92	65.7

**Vitamin D Receptor (VDR)*
***Others = GG*

Table 4. VDR genotype versus Sex

Crosstab					
			Sex		Total
			Male	female	
rs1544410		Count	4	4	8
		% within rs1544410	50.0%	50.0%	100.0%
		% within Sex	6.9%	4.9%	5.7%
	AG	Count	31	53	84
		% within rs1544410	36.9%	63.1%	100.0%
		% within Sex	53.4%	64.6%	60.0%
	GG	Count	23	25	48
		% within rs1544410	47.9%	52.1%	100.0%
		% within Sex	39.7%	30.5%	34.3%
Total		Count	58	82	140
		% within rs1544410	41.4%	58.6%	100.0%
		% within Sex	100.0%	100.0%	100.0%

Chi-Square Tests			
	Value	df	Sig. (2-sided)
Pearson Chi-Square	1.783[a]	2	0.410
Likelihood Ratio	1.778	2	0.411
N of Valid Cases	140		

Table 5. A-allele-in VDR versus Sex

Crosstab					
			Sex		Total
			Male	female	
A-allele-D	others	Count	23	25	48
		% within A-allele-D	47.9%	52.1%	100.0%
		% within Sex	42.6%	32.1%	36.4%
	A	Count	31	53	84
		% within A-allele-D	36.9%	63.1%	100.0%
		% within Sex	57.4%	67.9%	63.6%
Total		Count	54	78	132
		% within A-allele-D	40.9%	59.1%	100.0%
		% within Sex	100.0%	100.0%	100.0%

Chi-Square Tests					
	Value	df	Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	1.532[a]	1	0.216		
Continuity Correction[b]	1.111	1	0.292		
Likelihood Ratio	1.525	1	0.217		
Fisher's Exact Test				0.270	0.146
Linear-by-Linear Association	1.521	1	0.218		
N of Valid Cases	132				

Table 6. VDR VS PTH genotype

Descriptive

*D3						
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
					Lower Bound	Upper Bound
AG	35	30.7514	13.07329	2.20979	26.2606	35.2423
GG	92	29.9370	16.29544	1.69892	26.5623	33.3116
AA	5	26.2800	17.00682	7.60568	5.1632	47.3968
Total	132	30.0144	15.43799	1.34370	27.3562	32.6726

ANOVA

*D3					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	89.293	2	44.646	0.185	0.831
Within Groups	31132.150	129	241.334		
Total	31221.443	131			

** VitaminD3 level*

Table 7. VDR and A- allele

Group Statistics							
	A-allele-VDR	N	Mean	Std. Deviation	Std. Error Mean	p-value	95%CI
*D3	others	48	31.794	14.54360	2.09919	0.319	-2.73004 8.32230
	A	84	28.998	15.92201	1.73723		

A Allele A
Others GG
**D3VITAMIN D3 LEVEL*

Cross tabulation was also performed to examine association between genotyping and allele frequency against sex for PTH gene (rs11459015), AG percentage within sex is 29.3% in females group and 19% in males group, while GG percentage within sex is 62.2% and 70.7% in females and in males group, respectively. The percentage of AA within sex is 3.7% in females and 3.4% in males, but no association was found (p=0.560), moreover, allele A percentage within sex is 34.6 % in female and 24.1% in males also no association was detected (p=0.195). One way ANOVA was used to compare the mean of two or more variance between vitamin D3 serum levels and genotypes of PTH gene, but there was no statistical significance established (p=0.831) (Table 6). T test was carried out to test if there is any association between allele A and other (G) in VDR; thus, when comparing the mean of A allele (28.9976) to the mean of G allele (31.7937), the result was not statically significant (p=0.319) (Table 7).

Also T test was performed to test if there is any association between vitamin D3 levels and allele A in PTH gene, the mean of allele A is 30.1925 and other allele (G) is 29.9370, but that was statically not significant (p=0.931). Cross tabulation was carried out to test association of genotype within groups (patient and controls). For VDR gene (rs1544410); AG genotyping percentage within groups is 61.4% in patients and 58.6% in controls and GG genotype percentage is 38.6% in patients and 30% in controls, no AA genotype exist in VDR gene. The Chi square test shows dependency or association of AG genotype within the groups (p=0.012) (Table 8). Allele A percentage is 61.4% and 66.1% within patients and controls, respectively, but no significance was found (p=0.575). On the other hand, cross tabulation was performed in PTH SNP (rs1459015), gene to examine the association of genotyping within the groups (patients and controls). For AA genotype the percentage within groups is 4.3% in patients and 2.9% in controls, while AG genotype percentage is 30% in patients and

Table 8. VDR genotype versus cascont

Crosstab					
			cascont		Total
			control	case	
rs1544410		Count	8	0	8
		% within rs1544410	100.0%	.0%	100.0%
		% within cascont	11.4%	.0%	5.7%
	AG	Count	41	43	84
		% within rs1544410	48.8%	51.2%	100.0%
		% within cascont	58.6%	61.4%	60.0%
	GG	Count	21	27	48
		% within rs1544410	43.8%	56.3%	100.0%
		% within cascont	30.0%	38.6%	34.3%
Total		Count	70	70	140
		% within rs1544410	50.0%	50.0%	100.0%
		% within cascont	100.0%	100.0%	100.0%

Chi-Square Tests			
	Value	df	Sig. (2-sided)
Pearson Chi-Square	8.798[a]	2	0.012
Likelihood Ratio	11.890	2	0.003
N of Valid Cases	140		

Table 9. PTH genotype versus cascont

Crosstab					
			cascont		Total
			control	case	
rs1459015		Count	8	0	8
		% within rs1459015	100.0%	.0%	100.0%
		% within cascont	11.4%	.0%	5.7%
	AA	Count	2	3	5
		% within rs1459015	40.0%	60.0%	100.0%
		% within cascont	2.9%	4.3%	3.6%
	AG	Count	14	21	35
		% within rs1459015	40.0%	60.0%	100.0%
		% within cascont	20.0%	30.0%	25.0%
	GG	Count	46	46	92
		% within rs1459015	50.0%	50.0%	100.0%
		% within cascont	65.7%	65.7%	65.7%
Total		Count	70	70	140
		% within rs1459015	50.0%	50.0%	100.0%
		% within cascont	100.0%	100.0%	100.0%

Chi-Square Tests			
	Value	df	Sig. (2-sided)
Pearson Chi-Square	9.600[a]	3	0.022
Likelihood Ratio	12.701	3	0.005
N of Valid Cases	140		

Table 10. logistic regression

Variables in the Equation							
		B	S.E.	Wald	df	Sig.	Exp(B)
Step	Constant	0.121	0.174	0.484	1	0.487	1.129
Variables not in the Equation							
				Score		df	Sig.
Step 0	Variables		Sex	0.051		1	0.821
			Age	52.558		1	0.000
			D3	9.739		1	0.002
			AalleleD	0.314		1	0.575
		Overall Statistics		52.885		4	0.000
Omnibus Tests of Model Coefficients							
			Chi-square		df		Sig.
Step 1	Step		65.766		4		0.000
	Block		65.766		4		0.000
	Model		65.766		4		0.000
Variables in the Equation							
		B	S.E.	Wald	df	Sig.	Exp(B)
Step 1[a]	Sex	0.060	0.502	0.014	1	0.905	1.062
	Age	-0.177	0.033	29.330	1	0.000	0.838
	D3	0.003	0.017	0.023	1	0.880	1.003
	A-allele-D	-0.236	0.485	0.237	1	0.626	0.790
	Constant	7.127	1.500	22.584	1	0.000	1245.170
Variables in the Equation							
				95% C.I.for EXP(B)			
				Lower		Upper	
Step 1[a]			Sex	0.397		2.840	
			Age	0.786		0.893	
			D3	0.970		1.037	
			A-allele-D	0.305		2.043	
			Constant				

20% in controls and GG genotype percentage is 65.7% within patients and 65.7% within controls. Chi square test shows dependency or association of GG genotype within group (p=0.022) (Table 9). Allele A percentage within patient is 34.3% and 25.8% in controls, no statistical significance was established (p=0.290).

Logistic regression (results depicted in Table 10) was used to explain the disease by the explanatory variables that include sex, vitamin D3 serum levels and A allele of VDR gene. Significant of the model indicates its fitness to assess the relationship between all these variables. Odd ratio (OR) was greater than 1 in both sex and vitamin D variables, we could expect that sex (especially females) and level of Vitamin D both have an association with the SCD disease (each other), but they are not statistically significant (p=0.905) and (p=0.880) respectively OR of A allele (VDR) is relatively high (0.790), but not statistically significant (Table 10).

DISCUSSION

In this prospective cohort study, vitamin D3 serum levels observed in patients with SCD was lower compared to healthy subjects and the difference was statistically significant. Calcium (Ca), phosphate and ALP were showed statistical significance in favor of patients. On the other hand, fewer SCD patients had normal PTH levels compared to the controls and more patients had low PTH levels compared to controls; moreover, 10 patients had extremely high serum levels of PTH more than expected in relation to their low levels of vitamin D3, but that was not statistically significant. However, when those 10 patients knocked out (60 patient left) the difference became statistically significant for both PTH and vitamin D3. Secondary hyperparathyroidism could possibly be the only explanation for those 10 patients that showed shooting up of PTH serum levels as compensatory mechanism to keep the Ca level maintained, noted that most of patients showed normal Ca levels.

Accordingly, we would anticipated that these patients might have bone problems such as osteopenia or osteoporosis due to bone resorption or increased osteoclasts activity as a compensatory mechanism to maintain the balance of Ca and phosphate levels in the blood, as a consequence of this the bone mineral density (BMD) will be reduced, it would be very interesting to investigate the BMD in those SCD patients and see if supplements of Vitamin D3 could rectify BMD in SCD with abnormal BMD. Single nucleotide gene polymorphisms (SNP) had been carried out in this study to investigate if genotyping, allelic discrimination or allele frequency has any effects on SCD patients or phenotypes. To achieve this, two common SNPs were investigated, one in VDR gene (rs1544410 or Bsml) and the other one in PTH gene, (rs1459015), both SNPs are related to calcium metabolism(Jorde, Svartberg *et al*., 2012). G-allele is the ancestral allele for VDR gene. Our striking results in this study is that in VDR SNP (rs1544410) no mutant homozygous genotype with allele A (AA) was found in VDR gene neither in patients nor in controls. Our study showed that AG genotype is more frequent than the GG genotypes within each group and the difference was statistically significant. However, there is no statistical difference in the frequency of allele A between patients and controls. On the other hand, our analysis shows no association between genotypes and alleles frequency within sex. The females had higher percentage of AG genotypes compared to the males who had higher GG genotypes, but that was statistically not significant (p=0.410). However, the percentage of VDR Allele A is higher in females

(67.9%) compared to the males (57.4 %) and that was also didn't reach statistical significance (p=0.216). Regarding, the PTH gene (rs11459015) the G allele is the ancestral allele for this gene. In the current study association between genotypes and alleles frequency against sex was tested. We found that both AG and AA genotypes are more common in females than in males, but that was not statistically significant (p=0.560). Moreover, allele A frequency in patients is 40 (28.6%), but on splitting the patients group according to the sex, results revealed that the females had higher percentage of allele A compared to the males, but that was not statistically significant (p=0.195). The result of high frequency of AG genotypes among cohort leads us to assume that there could be an association between Vitamin D genotype/allele frequency and its phenotype. A hypothesis formed based on the assumption that allele A could have an association with vitamin D3 deficiency. Results revealed that the mean of allele A is (28.9976), while the mean for the other allele (G) is (31.7937).

Moreover, the fact that whenever there is vitamin D3 deficiency or insufficiency there is a high frequency of A allele in either patients or controls, but that was statistically not significant (p=0.319) this could probably due to low number of sample size. Therefore, the hypothesis that allele A in VDR gene could be associated with vitamin D3 deficiency should be clarified in very large group of patients or healthy subjects who have vitamin D deficiency. Opposite to our results in VDR gene our results in PTH gene revealed that the GG-genotype is more frequent than the AG genotype in both patients and controls and that reached statistical significance ($p < 0.022$). Allele A is more frequent within the patients (34.3%) than within controls (25.8%), but no statistical significance was established. The increased frequency of allele A in both patients and controls could be due to the presence of the AA genotypes. Odd ratio (OR) is greater than one "1" in both sex and vitamin D3 variables, we could anticipate that both sex (especially females) and levels of Vitamin D3 have an association with the SCD disease, but they are not statistically significant. Once more, OR of A allele (VDR) is relatively high (0.790), but that was not statistically significant.

Conclusion

More than 90% of our patients with sickle cell disease had vitamin D3 deficiency, which could certainly increase their chronic poor musculoskeletal health and possibly result in painful crisis. No significant association between any of the investigated genotypes and its phenotype. Thus, no statistical significance was shown between gene polymorphism of vitamin D3 or PTH and their serum levels, respectively. However, the complete absence of allele A (the mutant allele) in homozygous state in VDR gene in this study is shared between SCD and control subjects. Unfortunately, we could not be able to explain this observation, which might be due to our limited number of subjects included. Most of patients and controls that are deficient in vitamin D3 serum level carry allele A in a heterozygous state, accordingly hypothesis build on the assumption that allele A could have an association in some-way to the vitamin D3 serum level, but perhaps due to limited number of samples, hence more samples is needed to attain this hypothesis.

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