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ORIGINAL ARTICLE

Vitamin D Level and Vitamin D Receptor Gene Polymorphism as Predictors to Response to Bronchial Asthma Therapy in Children

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ABSTRACT

Background: Bronchial asthma (BA) is a serious worldwide health problem affecting all ages. Its frequency is increasing in many nations, particularly among children. Deficiency in Vitamin D and single nucleotide polymorphisms (SNP) in gene coding vitamin D receptor (VDR) have been associated with asthma. This study aimed to assess the role of serum vitamin level and VDR SNP fok1 rs 10735810 as predictors of therapy response in asthmatic children.
Methods: 96 subjects were included in the study; classified into **(**32 children with controlled bronchial asthma, 32 children with uncontrolled bronchial asthma and 32 healthy children). All children were subjected to serum vitamin D level measurement and Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) for VDR gene *FokI* polymorphism.
Results: There was statistical significant difference (P< 0.001) between the three studied groups as regard vitamin D levels**.** While, there was no statistically significant association (P≥0.05) between vitamin D levels and GINA treatment steps. Regarding *VDR FOK1*, there was statistically significant difference (P< 0.001) between the asthmatic and healthy control groups regarding the frequency of both Ff and FF genotypes with a p value of (0.027 & 0.002 respectively). Also, there was statistically significant association (P<0.05) between VDR polymorphism and GINA treatment steps.
Conclusions: The vitamin D deficiency was higher in children with controlled and uncontrolled asthma,in comparison to the healthy controls. There was a significant association between *FOK1* polymorphism and both association of asthma and GINA treatment steps. This suggests that VDR fok1 gene polymorphism might have a role in response to asthma treatment.

Keywords: Bronchial asthma; 25OHD ; *FOK1* ; vitamin D receptor.

INTRODUCTION

Bronchial asthma (BA) is a highly prevalent disease. It is considered a multifactorial disorder that arise from interaction between multiple genetic and environmental factors, the main pathology in asthma is chronic inflammation in the air way [1]**.** The immunomodulatory action of vitamin D has been greatly highlighted by researchers in the last years. Researches about vitamin D and incidence of asthma have presented conflicting results, however, it has been proved that the clinical status of asthmatic children has been improved by vitamin D supplementation [2] **.**Vitamin D is not just a micronutrient involved in calcium metabolism but has immune-modulatory function and has a role in pathogenesis of various inflammatory disorders; including BA. [3] Its signaling may alter the course of different inflammatory processes by regulating the expression of genes involved in production of pro-inflammatory mediators, interfering with transcription factors that have a role in regulating inflammatory genes or stimulation of signaling process mediating inflammation. [4**]**Vitamin D induces naïve T cell differentiation into regulatory T cell that secrete IL-10 and suppress dendritic cells that are considered fundamental in Th2 activation that has fundamental role in asthma development **[5]**.Vitamin D influences the immune system via its role on T helper cells(type 1 , 2 and regulatory T cells) and also through modulating chemokines produced by airway smooth muscle cells (ASM). So, serum vitamin D levels assessment and correcting recognized deficiencies may prevent the need for intensification of preventer therapy [6]**.**A previous study [7]

indicated that deficiency and insufficiency of vitamin D was of great significance in the initation and development of bronchial asthma in children , also, can induce allergic factors (IL 17 ,IgE), which are considered one of the risk factors in asthma .The biological action of vitamin D is exerted through its attachment to VDR which is one of nuclear receptor super family [8] and is found in many tissues beside bones and intestine involving immune cells (macrophages ,B cell, monocytes and T cell) and airway smooth muscle cells [9]**.** ASM cells have the enzymatic activity to convert 25-hydroxy vitamin D to 1, 25-dihydroxy vitamin D which in turn hinder its proliferation ,remodeling and inflammatory chemokines expression [10**]**Thus, the existence of VDRs on immune cells and several tissues in the airways demonstrate the role of vitamin D as an actually modifiable factor in asthma regarding its supposed immunomodulatory function [11].

In addition to the role of vitamin D in asthma development and severity, an association exists between vitamin D level and better response to inhaled ICS therapy for asthma **[**12]. It is observed that addition of vitamin D to steroids result in synergic effect on patient resistant to steroid therapy [13]. Also, a study **[**14] suggested that therapeutic response to ICS in asthmatic patient is increased by sufficient level of vitamin D.

VDR is encoded by VDR gene located at chromosome 12 at 12q 1314 and have six promotor area and eight exons (protein coding) [15] Numerous single nucleotide polymorphism (SNP) are identified in VDR gene, the most significant found in exon two (*FOK1, rs10735810*), intron eight (*ApaI and BsmI*) and last exon (*TaqI*) termed by restriction enzymes utilized for their detection **[**16]. *FOK1 SNP* situated in exon two results in replacement of thymine (T) by cytosine (C) at the 1st ATG site (ATG to ACG) which lead to the formation of three amino acid shorter protein that modulate the VDR expression **[**17]. The biological effects of vit D can be altered by *FokI SNP* due to its interference with transcription factors involved in the immune-inflammatory genes regulation [18].Possible correlation was found between vitamin D levels and VDR *SNP FOK1* F allele with requirement of therapy to obtain control of asthma. VDR might have important role in response to treatment of asthma and a likely mechanism to bypass this allelic variation is to raise vitamin D level as a compensatory tool [14]. The aim of this study was to assess the role of serum vitamin D level and VDR *SNP FOK1* as predictors of response to therapy in asthmatic children.

METHODS

The study was a case control study performed in Clinical Pathology and pediatric Departments, Zagazig University Hospitals. The patient samples were collected from pediatric clinics and pediatric department of Zagazig University Hospitals in the period from October 2017 to December 2018. Formal consent was obtained from all individual and the study protocol was approved by the Zagazig medical research ethical committee (ZU-IRB#3724-23-5-2017). The study was done according to The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans. 96 subjects were enrolled in this study; classified into three groups as **(**32 children with controlled bronchial asthma ,32 children with uncontrolled bronchial asthma and 32 healthy children).All children were subjected to full history taking, clinical examination and routine laboratory investigations; CBC by "Sysmex XS 500i" supplied by Sysmex Japan. Liver, kidney function tests and Vitamin D measurement on "Cobas 6000, e601 module , by electrochemiluminescence" supplied by Roche Diagnostic (Germany) ; ESR and specific investigation (DNA extraction and performance of PCR-RFLP VDR gene *FokI* polymorphism).

Inclusion criteria: Asthmatic children diagnosed by pediatric pulmonologist according to the global initiative for asthma (GINA) guidelines, Patients were on ICS regular treatment for at least 3 months from the time of diagnosis with or without long acting B2 agonist or leukotriene receptor antagonist. Patients with chronic diseases (e.g., cardiorespiratory, metabolic, or endocrine) were excluded

DNA extraction: The DNA was extracted from whole blood using the QIAamp DNA kit following the manufacturer's protocol (QiagenHilden, Germany) and was stored at -70°C.

Genotyping: DNA was amplified using MyTaq Red Mix composed of Ready- to-use 2x mix for fast and specific PCR. Primers were purchased as lyophilized agents. Forward primer **:**5'-AGCTGGCCCTGGCACTGACTCTGCTCT-3'and Reverse primer**:**5'-ATGGAAACACCTTGCTTCTTCTCCCTC-3'.

For each reaction the following reagents were added to each tube: Master mix 10 µl, Forward primer 1.0 µl, Reverse primer 1.0 µl, Template DNA 6.0 µl, Sterile high quality nuclease free water 22 µl.

The following temperature scheme was performed for the amplification using thermal cycler Biometra , An Analytik Jena Company (Germany) , Initial denaturation at 95C for one minute then 35 cycles of : -denaturation at 95C for 15 second ,annealing at 60 C for 15 second ,extension at 72 C for 10second ,final extension at 72 C for 7 minutes. PCR products were separated on a 2 % agarose gel at the band 265 bp with PCR

marker (ladder) 100 bp DNA ladder (Sibenzyme Ltd., Russia).The PCR products were stored at -20°C until used.The amplified DNA was digested by using *(FOKI)* restriction enzyme Thermo Scientific FastDigest FOKI 100µL (for 100 rxns). The following components were mixed at room temperature: (10 µl of the amplified PCR product ,17 µl of nuclease free water, 2 µl of 10x FastDigest Green Buffer and 1 µl of restriction enzyme) then incubated at 37°C for 5-15 minutes. The amplified fragment (265 bp) after being digested with *FOKI* restriction enzyme, was visualized on agarose gel 3% concentration, using ultraviolet transillumination gave rise to (figure 1) undigested fragment (265 bp) specified the presence of the F allele and appearance of 2 fragments (196 bp and 69 bp) specified the presence of the f allele. The homozygous mutant FF genotype led to one band at 265 bp, homozygous variant ff (wild type) led to two band s at 196 bp and 69 bp and heterozygous variant Ff led to three fragments at 196, 69 and 265 bp.

STATISTICAL ANALYSIS

The collected data were analyzed by Statistical Package for Social Science (SPSS) version 21. Qualitative data were represented as frequencies and percents. Chi square (X2) and Fisher's exact tests were used to detect relation between different qualitative variables. For quantitative variables mean, standard deviation, and (minimum and maximum) were computed. One way ANOVA test was used for detection of difference between different quantitative. The results were considered statistically significant and highly statistical significant when the significant probability (P value) was < 0.05* and <0.001** respectively.

RESULTS

Demographic and clinical characteristics of studied groups were summarized in table (1) Ensuring homogeneity of data as there was no statistically significant difference between controlled, uncontrolled asthmatic group and healthy control group as regard age or sex. Also, there was no statistically significant difference (P≥ 0.05) between controlled, uncontrolled asthmatic group and healthy control group as regard all laboratory findings except for Hb level, ESR and WBCs, there was highly statistical significant difference (P< 0.001) between them. The uncontrolled asthma group had the lowest Hb levels (9.79±0.76) followed by controlled asthma group (11.3±0.83) then the healthy controls (12.04±0.71). While ESR and WBCs were significantly higher among uncontrolled asthma group versus both controlled asthma and healthy controls groups (p<0.001).

There was highly statistical significant difference (P< 0.001) among the three studied groups regarding vitamin D levels as Mean± SD were 18.73±3.38, 16.30±6.46, 12.91±3.97 for the healthy controls, uncontrolled asthma and controlled asthma respectively (table 2).

There was no statistically significant association (P≥0.05) between vitamin D levels and GINA treatment steps while there was statistically significant association (P<0.05) between vitamin D levels and GINA control & different treatment among the asthmatic groups where controlled and corticosteroids only were more associated with deficient vitamin D level (table 3). There was statistically significant difference (P< 0.001) between asthmatic cases and healthy control as regard the frequency of risk allele F. The higher proportion of the two groups had FF genotype by a percent of 46.9% to control and 71.9% to cases. There was a statistically significant difference between cases and controls as regard the frequency of both Ff and FF genotypes with a p value of (0.027 & 0.002 respectively) (table 4).There was statistically significant difference (P< 0.05) between controlled, uncontrolled asthmatic group and healthy control group as regard ff and FF genotype while there was no statistically significant difference between them as regard Ff genotype. Using Pairwise comparison a significant difference was found between healthy control and both controlled and uncontrolled asthma groups (p = 0.029 and 0.002 respectively) with ff frequency higher in healthy control. While, there was no significant difference between uncontrolled asthma and controlled asthma regarding ff expression. A significant difference was found between healthy control and uncontrolled asthma as regard FF genotype frequency with a higher frequency among uncontrolled asthma. No significant difference was found between healthy control and controlled asthma or between uncontrolled asthma and controlled asthma as regard FF expression (table 4).There was statistically significant association (P<0.05) between VDR polymorphism and GINA treatment step. When doing Pairwise comparison there was a significance increase in the frequency of patients with Ff (43.5%) and FF (47.8%) genotype in step 3 versus ff genotype (8.7%). Moreover, A significant difference among step 4 patients as most cases in step 4 were of FF genotype by a percent of 93.8%, indicating the association between F allele and the requirement of higher doses of steroids. While, there was no statistically significant association between VDR polymorphism and other parameters (table 5).

Table 1: Demographic and clinical characteristics of studied groups

Variables	Healthy controls (n=32)	Uncontrolled asthmatic (n=32)	Controlled asthmatic (n=32)	P value
Age (years): Mean± SD	7.42±2.2	7.67±2.2	7.76±2.1	[a]0.804
Sex: Female Male	13 (40.6 %) 19 (59.4 %)	14 (43.7 %) 18 (56.3%)	17 (53.1%) 15 (46.9%)	[b]0.580
Hb	12.04±0.71	9.79±0.76	11.3±0.83	**<0.001****
	p1<0.001, p2<0.001, p3<0.001			
ESR	6.81±1.91	14.8±5.32	8.88±1.96	**<0.001****
	p1<0.001, p2<0.001, p3<0.001			
Platelets	258.6±71.9	255.3±73.2	251.2±74.3	0.921
WBCs	7.69±1.71	10.67±2.57	7.81±1.77	**<0.001****
	p1<0.001, p2=0.783, p3<0.001			
Total bilirubin	0.93±0.29	0.88±0.31	0.94±0.29	0.691
Direct bilirubin	0.16±0.05	0.16±0.06	0.16±0.06	0.889
Albumin	4.95±0.22	4.98±0.24	4.96±0.23	0.872
ALT	18.72±7.38	17.75±7.26	18.44±7.41	0.863
AST	22.41±6.69	22.59±6.95	22.75±6.80	0.980
Bun	6.25±1.55	6.31±1.62	6.31±1.62	0.984
S.creat	0.49±0.19	0.50±0.22	0.46±0.20	0.700

Table (2): Comparing mean levels of vitamin D (ng/ml) between the studied participants (n=96).

Variables	Healthy controls (n=32)	Uncontrolled asthmatic (n=32)	Controlled asthmatic (n=32)	P value
Vitamin D: Mean± SD Range - normal > 30 ng/ml - insufficient 20-30 ng/ml - deficient < 20 ng/ml	18.73±3.38 (13.1-24)	16.30±6.46 (8.5-23.5)	12.91±3.97 (5.9-24.3)	[a]**<0.001****

Table (3): Relation between vitamin D levels, GINA control, GINA steps of treatment and Treatment of the asthmatic groups (n=64).

Variable	Vitamin D		$^{a}X^{2}$	P value
	Deficient (48) No %	**Insufficient (16) No %**		
GINA control Uncontrolled(n=32) Controlled (n=32)	18 (56.3) 30 (93.7)	14 (43.7) 2 (6.3)	12	**0.001***
GINA steps Step 2 (n=9) Step 3(n=23) Step 4(n=32)	8 (88.8) 18 (78.2) 22 (68.7)	1 (11.2) 5 (21.8) 10 (31.3)	1.7	0.42
Treatment ICS only (n=12) ICS plus B2agonist or leukotriens (n=52)	12 (100) 36 (69.2)	0 (0.0) 16 (30.8)	4.92	**0.027***

[a] **Chi square test (X^2)**

Table (4): Comparison between the cases and controls as regard genotyping

	Control® (n = 32)	Cases (n = 64)	P	OR	95% CI	
					LL	UL
Genotypes						
ff	10 (31.2%)	4 (6.2%)	Reference			
Ff	7 (21.9%)	14 (21.9%)	0.027	5.03	1.15	21.8
FF	15 (46.9%)	46 (71.9%)	0.002	7.7	2.1	28.1
Alleles						
f	27(42.2%)	22(17.2%)	Reference			
F	37(57.8%)	106(82.8%)	<0.001	3.52	1.79	6.91
	Control® (n = 32)	controlled asthma (n=32)	Uncontrolled asthma (n=32)	p		**X^2**
ff	10 (31.2)	3(9.4)	1(3.1)	11.21		0.003*
p1=0.029*, p2=0.002*, p3=0.303						
Ff	7 (21.9)	9(28.1)	5(15.6)	1.46		0.481
P1= 0.0563, p2=0.521p3=0.226						
FF	15 (46.9)	20(62.5)	26(81.3)	8.18		0.016*
p1=0.209, p2=0.004*, p3=0.095						

[a] Chi square test (X^2), Pairwise comparison bet. each 2 groups was done using Mantel–Haenszel test
p: p value for comparing between the studied groups
p_1: p value for comparing between Healthy controls and controlled asthmatic
p2: p value for comparing between Healthy controls and unControlled asthmatic
p_3: p value for comparing between unControlled asthmatic and Controlled asthmatic

Table (5): Relation between VDR polymorphism, GINA control, GINA steps of treatment and Treatment of the asthmatic groups (n=64).

Variable	VDR			[a]X^2	[a]P value
	ff (4)	Ff (14)	FF (46)		
GINA control				2.92	0.231
Uncontrolled(n=32)	1 (3.1)	5 (15.6)	26 (81.3)		
Controlled (n=32)	3 (9.4)	9 (28.1)	20 (62.5)		
GINA Treatment steps					
Step 2 (n=9)	1 (11.1)	3 (33.3)	5 (55.6)	3.76	0.152
Step 3(n=23)	p1=0.257, p2=0.045*, p3=0.342				
Step 4(n=32)	2 (8.7)	10 (43.5)	11 (47.8)	10.8	0.004*
	p1=0.007*, p2=0.003*, p3=0.767				
	1 (3.1)	1 (3.1)	30 (93.8)	15.5	**<0.001****
	p1=1.0, p2<0.001**, p3<0.001**				
Treatment					
ICS only (n=12)	1 (8.3)	3 (25)	8 (66.7)	0.22	0.894
ICS plus B2agonist or leukotriens (n=52)	3 (5.8)	11 (21.2)	38 (73)		

[a] Chi square test (X^2), Pairwise comparison bet. each 2 groups was done using Mantel–Haenszel test
p: p value for comparing between the studied groups
p_1: p value for comparing between ff and Ff
p2: p value for comparing between ff and FF
p_3: p value for comparing between Ff and FF

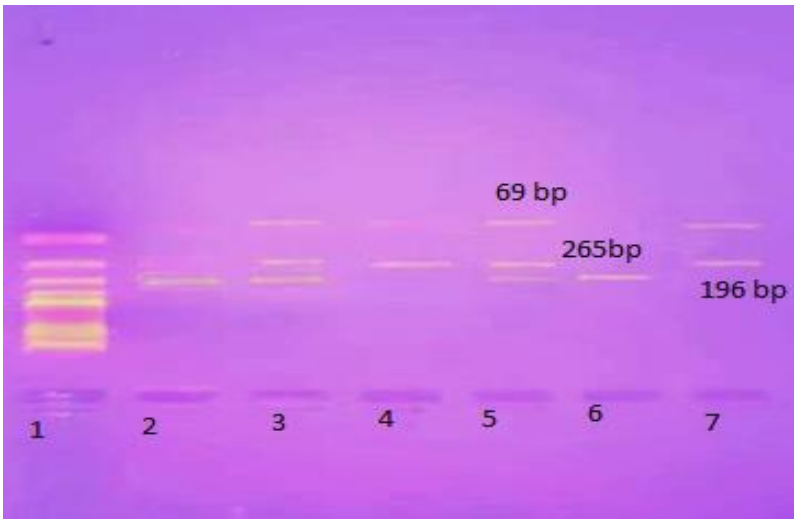

Figure (1): Gel electrophoresis of PCR-RFLP technique of amplified FOK1 genotypes.Lane 1 : ladder(100 bp), Lane2,6: represent homozygous mutant FF genotype showing one fragment at 265bp, Lane 3 and 5: heterozygous Ff showing three fragments at 265,196 and 69 bp., and Lane 4,7: represent homozygous wild ff genotype showing two fragments at196 bp and 69 bp.

DISCUSSION

The immunomodulatory action of vitamin D has been greatly highlighted by researchers in the last years. Many researches about vitamin D and incidence of asthma have presented conflicting results, however, it has been proved that the clinical status of asthmatic children has been improved by vitamin D supplementation [2]

This study is a case control study that included asthmatic children (controlled and uncontrolled) and healthy controls. The aim of this study was to assess the role of serum vitamin D level and VDR SNP fok1 as predictors to response to therapy in asthmatic children. In the current study, there was statistically significant difference among studied groups regarding vitamin D level, with the asthmatic groups (controlled & uncontrolled) having lower vitamin D levels. This finding is in agreement with several studies [19-24]who showed that the levels of serum vitamin D in the asthmatic children was significantly lower than that in the non-asthmatic children. In contrast, other studies [7, 25] found no statistically significant difference in vitamin D levels between the asthma group and the control groups .

The low levels of vitamin D in asthmatic children may be related to the lifestyle changes as they spent more time in indoor areas due to exacerbation and for fear of infections and as a result they were exposed to less sunlight [26]. However, recent findings regarding the role of vitamin D in bronchial asthma referred to the immunomodulatory effects of vitamin D on inflammatory cells in allergic asthma so vitamin D deficiency might be a cause rather than a result [27]. In the current study, the uncontrolled asthmatic group had higher vitamin D levels than controlled asthmatic groups. This finding is in agreement with two previous studies [14, 20] Who reported that serum 25(OH)D levels were significantly higher in uncontrolled compared to controlled patients with asthma. On the other hand, several studies [28-30] showed that lower vitamin D levels were associated with poor asthma control. One possible explanation for this discrepancy is that the most important element of the treatment is inhaled corticosteroids, and the dose of inhaled corticosteroid increases as well as the severity of the disease increases, too many studies have presented that the corticosteroids use may decrease the serum vitamin D levels in patients with asthma. [31, 32] So, including the information on the duration and dose of inhaled corticosteroid use might clear the conflict in these results and clarify whether vitamin D deficiency is a cause of severity of asthma or is it a result of treatment.

Regarding the present study, there was no statistically significant association between vitamin D levels and GINA treatment steps. This is in line with a previous study [33]but partly against another study [14] that observed a higher proportion of vitamin D in uncontrolled asthmatic patients at step 4 of the GINA treatment in comparison to controlled asthmatic patients. This discrepancy with our result may be due to the fact that vitamin D deficiency became common problem in our country even in healthy people.

Regarding vitamin D levels in asthmatic groups, there was statistically significant association between vitamin D levels & different treatments among the asthmatic groups where those controlled on corticosteroids only were more associated with

deficient vitamin D level. This finding was opposed by Einisman et al. [14] ,whose results did not show any significant association between vitamin D levels and different treatment among asthmatics. However, several studies have provided evidence that steroid therapy leads to reduction in serum level [31, 32],indicating that long term use of steroid therapy might be a cause of vitamin D deficiency. So the higher frequency of vitamin D deficiency in controlled asthma group on steroid alone might be attributed to the higher doses of steroids used to reach control of asthma when used alone. Comparison of VDR gene polymorphism showed that a higher proportion of all groups had FF genotype. However, there was statistically significant difference between patients and controls, with a higher frequency of FF and Ff genotypes in the asthmatic group. Our results suggested that F allele is a risk allele for asthma as 82.8% of asthmatic children were carriers of F allele versus 57.8% among the control group.

Moreover, there was a higher frequency of FF genotype carriers among uncontrolled asthma group (81.3%) compared to controlled asthma (62.5%) and healthy control (46.9%) groups. However, there was no significant association between *VDR FOK1* genotypes and vitamin D level. Matching with our result, a study [24] found that *VDR fok1* polymorphism FF would be considered as a potential risk factor for asthma in the Kurd ethnicity. Also, these results matched with a meta-analysis revealing that *Fokl* polymorphism (C>T) may be related to asthma in white pediatric patients **[34]**. Also, a study on Egyptian asthmatic children [35] showed that children having the F allele are nearly 2 and a half times as susceptible to the development of asthma than healthy individuals.A systematic meta-analysis of 17 case control studies was conducted to assess VDR gene polymorphism and susceptibility to asthma. The results of pooled analysis revealed a statistical significant association between *FokI* SNP with asthma risk [18]. In contrast, several studies [25, 36]found no significant difference between the asthma and control groups in terms of *FokI* polymorphism. In addition, others [15, 37, 38] reported that FF genotype of *FokI* was less in asthmatic patients compared to healthy control. Other factors, such as ethnicity, geographical features of the place, climate, and lifestyle, may explain the contradicting findings of different VDR SNP studies [39].Also, the present study found a statistically significant association between FOK1 polymorphism and GINA treatment step which supports the hypothesis that VDR may have a role in asthma treatment response as most patients on GINA treatment step 4 (93.7%) were (*FokI VDR SNP FF*) in comparison to asthmatics in step 2 and 3 (55.6% and 47.8% respectively) . These findings point out the association between FF genotype and the requirement for higher doses of steroid

This finding is in line with another study [20] **,** who found an association between F allele and glucocorticoid resistance. Moreover, a study [14] found a possible association of *FokI* and GINA treatment groups, as all patients on step 4 of treatment were having the C allele, Thus there was a possible association between *FokI* C allele and higher requirement of therapy to reach asthma control, suggesting that it may be involved in treatment response. Several VDR SNPs alter vitamin D function, so hindering its activity, even in individuals with normal vitamin D levels. The VDR gene had been classified one of the candidate genes of asthma by multiple studies, However, few studies have addressed the relationship between VDR SNP and response to therapy among with asthmatic patients [20, 40].Vitamin D receptor has a vital influence on metabolism of calcium, which had a role in vitamin D levels regulation through feedback mechanism, which in turn may explain our finding. Also, another type of feedback may be exerted by VDR to overwhelmed this deficient activation, thus, it could be a possible explanation for uncontrolled patients to have higher levels of vitamin D. If these theories were proven true, it would be suggest that parenteral vitamin D administration could overcome this deficient activation and aid to reach a well response to treatment in genetically predisposed patients [14].

CONCLUSIONS

Given the results of the present study, we can conclude that the frequency of vitamin D deficiency was higher in children with controlled and uncontrolled asthma, compared to the healthy control. Moreover, F allele was considered a risk allele for development of asthma in children. Also, there was a significant association between VDR polymorphism *FOK1* and GINA treatment step. This suggests that VDR polymorphism *FOK1* might have a role in asthma treatment response

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