Vitamin D receptor gene polymorphisms in Egyptian children with bronchial asthma, A case-control study.

[1]Amira Ahmed Abdel Wahab
[1]Assistant Lecturer
Department of Pediatrics
Faculty of medicine- Cairo University
Cairo-Egypt
doc_amora@hotmail.com

[2]Hala Hamdi Shaaban
[2]Professor
Department of Pediatrics
Faculty of medicine- Cairo University
Cairo-Egypt
hhaitham1964@yahoo.com

[3]Abeer Mohamed Mohy
[3]Professor
Department of Clinical Pathology
Faculty of medicine- Cairo University
Cairo-Egypt
ashour572@hotmail.com

[4]Noussa Ragab Mohamed
[4]Professor
Department of Pediatrics
Faculty of medicine- Cairo University
Cairo-Egypt
noussaelbasha3@yahoo.com

[5]Nihal Mohamed Elrefaai
[5]Professor
Department of Pediatrics
Faculty of medicine- Cairo University
Cairo-Egypt
nihal-r@hotmail.com

[6]Douaa El Saied El Sherbiny
[6]Lecturer
Department of Pediatrics
Faculty of medicine- Cairo University
Cairo-Egypt
doaa.sherbiny@gmail.com
ORCID ID: 0000-0002-6627-3701
Corresponding author

***Abstract*:**

Background: Vitamin D is known for its potential anti-inflammatory effect in inflammatory diseases such as bronchial asthma. Thus, vitamin D receptor gene polymorphism may contribute to genetic susceptibility to asthma. There is a paucity of knowledge on vitamin D receptors, single nucleotide polymorphism (SNP) in Egyptian asthmatic children.

Methods: We enrolled children; aged from 2-12 years; 100 asthmatic and 100 healthy. They were investigated using a complete blood picture, total serum IgE level, and DNA analysis using PCR RFLP technique for identification and analysis of SNP, FokI (rs 2228570), and BsmI (rs 1544410).

Results: Analysis of possible genetic models showed a significant difference between cases and control in the recessive model of FokI SNP (Ff & ff), (p=0.04), as well as the (f) allele (p=0.02). On the other hand, genotypes and allele frequencies of BsmI SNP were not significantly different between asthmatic children and control (P=0.24 & 0.35 respectively). Both SNPs were not significantly different among the three groups of asthma severity (mild, moderate, and severe persistent).

Conclusions: The recessive model of FokI SNP (Ff & ff), as well as the presence of the f allele, were independently associated with an increased risk of asthma in Egyptian children.

Keywords: Vitamin D receptor- gene polymorphism- Bronchial asthma- Children

I. INTRODUCTION

Bronchial asthma (BA) is a chronic inflammatory airway disease that is influenced by the potential anti-inflammatory effect of vitamin D. Vitamin D modulates the inflammatory response through regulation of pro-inflammatory cytokines, interference with transcription factors involved in inflammation as well as activation of cascades that mediate inflammation[1]. This effect is exerted by binding the active form of vitamin D; 1,25-dihydroxycholecalciferol (1,25 (OH)2 D3) to the Vitamin D receptor (VDR), which in turn is coded by the VDR gene[2]. Some of the single nucleotide polymorphisms (SNPs) in the VDR gene affect VDR protein synthesis (e.g., FokI), while others affect mRNA stability and gene transcription (e.g., BsmI). Thus, vitamin D and SNP in the VDR gene are linked to the complex genetic susceptibility to bronchial asthma [3], [4]**.** Nuclear receptors with anti-inflammatory effects such as VDR explains the prevalence of BA in certain individuals in a specific population and may constitute promising targets for the treatment of BA[5]**.**

Several studies have investigated the association between VDR gene polymorphism and asthmatic populations in different ethnic groups. The most studied SNP in the VDR gene were FoKI, BsmI, ApaI, and TaqI. Our study aimed to investigate the possible association of certain VDR gene SNP; FokI and BsmI genotypes & alleles with bronchial asthma in Egyptian children. We also aimed to detect the impact of these polymorphisms on asthma severity, atopy, absolute eosinophilic count, and total serum immunoglobulin E among asthmatic children.

II. MATERIALS AND METHODS

A. Study design and population

We conducted this case-control study at the outpatient clinics of Children's Hospital, Cairo University. We considered children eligible for the study if their ages ranged from 2-12 years. We excluded children from the study if they had: chronic chest diseases other than asthma, gastroesophageal reflux disease (GERD), cardiac diseases, any systemic diseases, or a history of systemic steroids intake within 2 weeks before sampling. We divided the enrolled children into two

groups. The asthmatic group included one hundred children following at the allergy clinic, being diagnosed with asthma, and classified according to asthma severity, based on the global initiative for Asthma (GINA) guidelines 2015. The control group included one hundred (age and sex)- matched children who sought medical advice at the general clinic and have no personal or family history of asthma or other atopic manifestations.

B. Diagnostic procedures

All enrolled children were subjected to full history taking and clinical examination. We assessed asthma severity for asthmatic children. Four milliliters of venous blood were withdrawn and collected from each participant: 1 ml in an EDTA tube for Complete blood count assay, 1 ml in a plain tube for total serum IgE level assay, and 2ml in sterile EDTA vacutainers for DNA extraction and analysis. Samples were stored at -20◦C until the time of assay.

Total serum IgE was determined using a human IgE ELISA kit (quantitative solid phase sandwich enzyme immunoassay technique) provided by IDLabsTM Biotechnology Inc.(http://www.idlabs.com).

Genomic DNA was extracted from peripheral blood leukocytes of EDTA anticoagulated blood using "QIAamp DNA Blood Mini kit; Qiagen GmbH Hoffmann-La-Roche AG, Hilden, Germany". Enzymatic amplification was performed by PCR using " Dream TaqTM Green PCR Master Mix (supplied by Fermentas UAB, Vilnius, Lithuania) and Hybrid thermal cycler (Promega Corporation, Madison WI, USA)", following Folwaczny et al., 2005 protocol [6]The PCR reaction mixture (20 µl) contained 10 µl 2x PCR master mix [10 x PCR buffer, 4mmol/L $MgCl_2$, 0.5 units Taq DNA polymerase/µL, and 400 µmol/L of each deoxyribonucleotide triphosphate (dNTP) (dATP, dCTP, dGTP, dTTP)], 1 µl for 5′ forward primer, 1µl for 3′ reverse primer, 3 µl of genomic DNA and 5 µl of sterilized nuclease-free water. Analysis of DNA was done for two vitamin D receptor gene polymorphisms; FokI and BsmI: using PCR-RELP (restriction fragment length polymorphism) using two sets of primers for each. The reaction was carried out with the following cycles: 95ºC for 5 minutes, 35 cycles (30 s denaturation at 95ºC, 30 s annealing at 55ºC and 1-minute extension at 72ºC), and 10 minutes final extension at 72ºC after completion of the cycles. Amplified samples were then run in parallel on 1.5% agarose gel containing ethidium bromide using gel electrophoresis and visualized by UV transillumination. Amplified products were digested with FastDigest restriction enzyme at 65% for 10 minutes and digested products were detected on 3.5% agarose gel electrophoresis containing ethidium bromide, (table 1).

C. Outcomes

Our primary outcome was the detection of FokI and BsmI genotypes & alleles associated with bronchial asthma in children. The secondary outcome was the possible association of FokI and BsmI genotypes & alleles with the severity of asthma, atopy, absolute eosinophilic count, and total serum IgE.

Table 1. Loci selected in the vitamin D receptor sequence and their primers

SNP	PCR primer*	PCR product (bp)	Restriction enzyme#		RELP products (bp)
			Prepared from	Recognition sequence	
FokI	F: 5'-AGC TGG CCC TGG CAC TGA CTC TGC TCT-'3 R: 5'-ATG GAAACA CCT TGC TTC TTC TCC CTC-'3	265-bp	Flavobacterium okeanokoites, a gram negative bacteria.	5'-GGATG N9↓-3' 3'- CCTAC N13-5'	FF: 265bp Ff: 265, 196 and 69bp ff: 196 and 69bp
BsmI	F:5'CAACCAAGACTAC AAGTACCGCGTCAGTG A-'3 R:5'-AACCAG CGGGAA GAG GTCAAGGG-'3	825-bp	Bacillus stearothermophilus, a gram positive bacteria.	5' GAATGC N↓ 3' 3' CTTAC↑GN 5'	BB: 825bp Bb: 825, 650 and 175bp bb: 650 and 175bp

∗ Primers were provided by Operon Biotechnologenices GmbH/BioCampus, Cologne Nattermannallee 1/ 50829 Cologne, Germany
F:forward, R: reverse. **#** FokI and BsmI enzymes were supplied by NewEngland BioLabs

D. Statistical Method

Data were coded and entered using the statistical package SPSS version 16. Data were analyzed using numbers and percentages for qualitative variables, mean and Standard deviation for normally distributed quantitative variables, and median and interquartile range for non-normally distributed quantitative variables. Comparison between groups was done using the chi-Square test for qualitative variables, analysis of variables (ANOVA) and independent sample (t-test) for normally distributed quantitative variables, non-parametrical Kruskal-Wallis test, and Mann-Whitney test for non-normally distributed quantitative variables. Correlations were done to test for linear relations between variables. A p-value less than or equal to 0.05 was considered statistically significant.

III. RESULTS

A total of 200 children were enrolled, in two groups; Group 1 included 100 asthmatic children and Group 2 (control) included 100 healthy children. We started recruiting children in October 2015, and the primary completion date was March 2017. Among asthmatic children, the most common precipitating factors were passive smoking (49%) and infection (39%). Mild persistent asthma was the predominant type (40%), followed by moderate persistent asthma (36%) and severe persistent asthma (24%). Atopic manifestations were present in 36% of cases, while positive family history of atopy was present in 51% of cases. The most common asthma medications received were inhaled B2 agonists (62%), inhaled steroids (52%), and oral B2 agonists (39%), (table 2).

Table 2. Characteristics of enrolled asthmatic children (n=100)

		n (%)
Asthma severity	Mild Persistent	40(40%)
	Moderate Persistent	36(36%)
	Severe Persistent	24(24%)
Precipitating factors	Passive smoking	49(49%)
	Infection	39 (39%)
	Food	19(19%)
	Others (dust, exercise, smoke)	24(24%)
Seasonal variation	Winter	64 (64%)
	Summer	12 (12%)
	Perennial	10 (10%)
	Perennial with seasonal flare	14 (14%)
Medications	Inhaled Beta 2 agonist	62(62%)
	Inhaled corticosteroids	52(52%)
	Oral B2 agonist	39(39%)
	Leukotrienes receptor antagonist	32(32%)
	Mast cell stabilizer	29(29%)
Atopy	Atopic manifestations*	36(36%)
	Family history of atopy	51(51%)

*Conjunctivitis, dermatitis, rhinitis

Both asthmatic and control groups were similar regarding their age, sex, haemoglobin, total leukocytic count, and platelet count. The medians (IQR) for the absolute eosinophilic count and total serum IgE tended to be higher among asthmatics, but the difference was not statistically significant, (table 3).

Table 3. Demographics and investigations in asthmatic and control group

		Asthmatics (n=100)	**Control (n=100)**	**P-value**
Demographics	Age (years)	6.69±2.82	6.06±2.91	0.74
	Males	59 (59%)	68(68%)	0.19
Investigations	Hemoglobin (g/dl)	12.34± 1.01	12.14±1.29	0.23
	Platelets ($x10^3$/ mm^3)	301.89±92.76	307.92±82.74	0.63
	Total leucocytic count ($x10^3$/ mm^3)	8.66±3.14	8.53±4.1	0.26
	Absolute eosinophilic count (/mm^3)	175(66.2-399)	97(47-300)	0.16
	Total serum IgE (IU/L)	87(40-236)	60(30-160)	0.09

Categorical variables are given as n (%) while continuous variables are given as mean ± SD or median (IQR)

A. Vitamin D receptor (VDR) single nucleotide polymorphism (SNP) among all enrolled children (n=200)

Regarding FokI, the most common genotypes were the reference (FF) and the heterozygous (Ff), (47% and 45.5% respectively), while the homozygous genotype (ff) was detected only in 7.5%. For BsmI genotypes, the heterozygous (Bb) represents the majority; 61.5%, followed by the reference (BB)

which represents 29%, while the least common was the homozygous (bb), being detected only in 9.5 % of enrolled children.

B. VDR SNP among asthmatic versus control groups

For FokI, the frequency of (FF, Ff & ff) genotypes tended to be different in both groups, with a p-value close to significant (p=0.053). Further analysis showed that the frequency of the combined Ff & ff genotypes was significantly higher among asthmatics when compared to the control group (p = 0.047), and the f allele was more prevalent in asthmatics (p=0.02). The reference genotype FF (versus ff) was more prevalent in the control group (p=0.027). For BsmI, there was no statistically significant difference between all genotypes and alleles among both groups, (table 4).

Table 4. Vitamin D receptor gene polymorphism (FokI and BsmI) among asthmatics and control

		Asthmatics (n=100)	**Control (n=100)**	**OR**	**95% CI**	**P-value**
FokI						
Genotypes	Reference (FF) Heterozygous (Ff) Homozygous (ff)	40(40%) 49(49%) 11(11%)	54(54%) 42(42%) 4(4%)			0.0 53
Models	Recessive (Ff+ff vs FF) Homozygous (FF+ff vs Ff) Dominant (FF+Ff vs ff) Codominant 1(FF vs ff) Codominant 2(FF vs Ff) Codominant 3(ff vs Ff)	60 (60%) 58 (58%) 89 (89%) 40/51 (78%) 40/89 (45%) 11/60 (18%)	46 (46%) 51 (51%) 96 (96%) 54/58 (93%) 54/96 (56%) 4/46 (9%)	1.76 1.33 2.97 3.71 1.58 0.42	(1.005-3.086) (0.759-2.318) (0.911-9.655) (1.101-12.515) (0.881-2.815) (0.126-1.432)	0.047* 0.32 0.06 0.027* 0.12 0.15
Alleles	F allele f allele	129/200(64.5%) 71/200 (35.5%)	150/200(75%) 50/200 (25%)	1.65	(1.072-2.542)	0.02*
BsmI						
Genotypes	Reference genotype (BB) Heterozygous genotype (Bb) Homozygous genotype (bb)	28(28%) 59(59%) 13(13%)	30(30%) 64(64%) 6(6%)			0.24
Models	Recessive (Bb+bb vs BB) Homozygous (BB+bb vs Bb) Dominant (BB+Bb vs bb) Codominant 1(BB vs bb) Codominant 2(BB vs Bb) Codominant 3(bb vs Bb)	72 (72%) 41 (41%) 87 (87%) 28/41 (68%) 28/87 (32%) 13/72 (18%)	70 (70%) 36 (36%) 94 (94%) 30/36 (83%) 30/94 (32%) 6/70 (9%)	1.1 0.81 2.34 2.32 0.99 0.43	(0.598- 2.031) (0.458-1.432) (0.852-6.430) (0.776-6.945) (0.529-1.845) (0.152-1.192)	0.76 0.47 0.09 0.13 0.97 0.10
Alleles	B allele b allele	115/200(57.5%) 85(42.5%)	124/200(62%) 76(38%)	1.2	(0.808- 1.799)	0.35

*Significant p-value. Categorical variables are given as n (%)

C. VDR SNP among asthmatic children

Regarding asthma severity, there were no statistically significant differences in comparing FokI and BsmI genotypes between different subgroups of severity. Regarding atopy, studying FokI genotypes showed that the homozygous variant (ff) tended to be higher in the positive than negative atopic group (19.5% versus 6.3% respectively) but the difference was not statistically significant. For BsmI

genotypes, although the heterozygous genotype (Bb) was slightly higher in atopic than non-atopic asthmatics (69% vs 53%), while other genotypes were slightly higher in non-atopic than atopic asthmatics (BB 31% vs 22%, bb 16% vs 8%), there was no statistically significant difference. Regarding eosinophilic counts, there were no statistically significant differences upon comparing FokI and BsmI genotypes. Regarding IgE, FokI genotypes, the median of total serum IgE level in the (ff) genotype (150 IU/L) tended to be higher than IgE in other genotypes FF & Ff (85 & 74 IU/L respectively), without statistically significant difference. For BsmI genotypes, although the IgE level was higher in the reference genotype (BB); 144 IU/L than in other genotypes (Bb; 80 IU/L and bb; 83 IU/L), the difference was not statistically significant, (table 5).

Table 5. Asthma severity, atopy, absolute eosinophilc count and total serum IgE in different vitamin D receptor gene polymorphism (Fok I and BsmI) among asthmatic children (n=100)

	Asthma Severity				**Atopy**			**Absolute eosinophilic count**		**Total serum IgE**	
	Severe persistent n=24	**Moderate persistent n=36**	**Mild persistent n=40**	p-value	**Positive n=36**	**Negative n=64**	p-value	**cells/mm3**	p-value	**IU/L**	p-value
FokI											
FF Ff ff	8 (33%) 12(50%) 4 (17%)	18 (50%) 14(39%) 4 (11%)	14 (35%) 23 (58%) 3 (8%)	0.42	13 (36 %) 16(44%) 7(20%)	27 (42%) 33(52%) 4(6%)	0.13	196(84-371) 120(21-269) 186(60-462)	0.21	85 (47-314) 74(38-252) 150(40-242)	0.71
Ff & ff	16(67%)	18(50%)	26 (65%)	0.31	23(64%)	37(58%)	0.55	126(49-387)	0.2	87.4(40-241)	0.9
BsmI											
BB Bb bb	6 (25%) 17 (71%) 1 (4%)	6 (17%) 23(64%) 7 (19%)	16 (40%) 19 (48%) 5 (13%)	0.09	8(22%) 25(70%) 3 (8%)	20(31%) 34(53%) 10(16%)	0.27	121(42-250) 176(85-333) 240(31-490)	0.4	144(61-248) 80(25-259) 83(53-355)	0.3

FF & BB: reference genotypes, Ff & Bb: heterozygous genotypes, ff & bb: homozygous genotypes. Data are given as n (%) and median (IQR)

IV. DISCUSSION

The association of VDR gene polymorphism with susceptibility to asthma has been studied in different populations. We aimed to study two VDR polymorphisms (FokI and BsmI) to find out if they are linked to asthma susceptibility in our population.

Our study showed that the three FokI genotypes (FF; reference, Ff; heterozygous, ff; homozygous) tended to be different in asthmatic and control groups, (p=0.053). Further analysis showed that the recessive model of FokI SNP (Ff & ff) was significantly higher in asthmatic children when compared to controls (P=0.047, OR.1.76; 95% C.I. (1.005–0.86). Moreover, the f allele was more prevalent in asthmatics (P=0.02, OR=1.65), which signifies the role of this allele in increasing the risk of asthma. This agrees with two African studies; on Tunisian and Egyptian children as well as a study on Serbian adults, that found a significant difference between asthmatic and healthy individuals regarding the FokI genotypes and alleles [3] [7] [8] Moreover, a recent meta-analysis concluded that FokI SNP was significantly related to the risk of asthma in f allele versus F allele, (P = 0.015) as well as in ff+Ff versus FF (P = 0.004) among Caucasians [9] These findings disagree with three studies on Chinese population and a meta-analysis of different populations that showed no difference between

asthmatic and control groups regarding FokI genotypes and alleles [10][11][12].

On the other hand, the BsmI genotypes (BB; reference, Bb; heterozygous, bb; homozygous), alleles (B, b) and possible models showed no statistically significant difference between asthmatic and control groups. These findings agree with studies on Cypriot adolescents, Serbian adults and Chinese children [3] [13] [14]. In contrast to our findings, the study on Tunisian children showed a statistically significant difference between asthmatics and controls regarding BsmI genotypes and alleles (P = 0.006 & 0.04 respectively), where children with homozygous 'B' allele were 2.15 times more likely to have asthma than others [7]. Also, a meta-analysis found that a codominant model of BsmI (BB vs bb) was significantly associated with asthma risk (p = 0.017) [12].

Upon studying our random sample of asthmatic children, male to female ratio was 3:2, which reflects the predominance of asthma before puberty in males [15], [16], owing to their smaller airway diameters relative to lung volumes compared to girls [17]

When asthmatic children were divided into subgroups based on asthma severity, there was no statistically significant difference in FokI genotypes (p= 0.42), which agrees with the study on Tunisian children (p=0.9) [7]. Also, among these subgroups, the frequency of the three BsmI genotypes showed no special pattern of association (P=0.09), which agrees with a study on Chinese children that did not find a correlation of BsmI with lung function levels; one of the criteria of asthma severity [13].

When asthmatics were divided into subgroups based on the presence of atopy, there was no statistically significant difference in FokI genotypes, apart from a tendency to a higher percentage of ff genotype in positive atopic when compared to negative atopic asthmatics (p= 0.13). On the other hand, Nabih & Kamel's study on the Egyptian population found that FF genotype was significantly higher among non-atopic versus atopic asthmatic (p = 0.041) as well as in healthy versus atopic asthmatic children (p = 0.002) [8]. For BsmI genotypes, the heterozygous genotype (Bb) tended to be higher in atopic than non-atopic asthmatics, while other genotypes (BB, bb) tended to be higher in non-atopic than atopic asthmatics, without statistically significant difference. This agrees with the Tunisian study which did not find any correlation between BsmI genotype and atopy in asthmatics [7] On the other hand, Heine et al found that the VDR haplotype bCT (the three alleles: BsmI b, Apal C and Taql T) was correlated with severe Atopic dermatitis in German adults [18]

Also, we observed that total serum IgE was slightly higher in the ff genotype when compared to other genotypes, but without statistically significant difference (P=0.710). However, Nabih & Kamel found that the highest IgE level was detected in the ff genotype with a statistically significant difference [8], probably due to the production of a longer VDR protein when compared to the FF genotype (427 versus 424 amino acids respectively). The longer VDR protein has a lower affinity for 1,25(OH)2D3 binding and lower transcriptional activity [19]. Also, the IgE level tended to be higher in the reference genotype (BB), compared to other BsmI genotypes, without statistically significant differences. However, Raby et al. observed significant associations between multiple VDR polymorphisms and immunoglobulin E levels [5].

The population of our study was geographically different from other comparative studies and was exposed to different environmental triggers. This may explain why our results differ from results carried out in different populations. Inadequate sample sizes, clinical heterogeneity, statistical power, or different combinations of these factors may also result in discrepancies in findings between the studies.

V. CONCLUSIONS

Our study revealed that the recessive model of FokI SNP (Ff & ff), as well as the presence of the f allele, is independently associated with an increased risk of asthma in our population. The BsmI genotypes and alleles are not linked to the risk of asthma. Moreover, there is no association between certain FokI and BsmI, genotypes or alleles with asthma severity, atopy, absolute eosinophilic count and total serum IgE in asthmatic children.

More studies with a larger sample size will improve our understanding of the contribution of these two genes and other genes to asthma status and atopy. This may allow the development of new preventive and/or therapeutic interventions in asthmatic patients.

VI. LIST OF ABBREVIATIONS

BA: bronchial asthma,
VDR: Vitamin D receptor,
SNPs: single nucleotide polymorphisms,
GERD: gastroesophageal reflux disease,
GINA: global initiative for asthma,
dNTP: deoxyribonucleotide triphosphate.

VII. DECLARATIONS

All experimental protocols were approved by the ethical standards of the "Ethics Committee of Cairo University". Written informed consent was obtained from a parent and/or legal guardian of all participants before enrolment by the principal investigator. The authors declare that they have no competing interests. The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

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