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Effect of Vitamin D Binding Protein (DBP) Genotype on the Development of Asthma in Children

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Introduction

Recently there has been extensive interest in “non classical” functions of vitamin D, in contrast to the classical role of vitamin D in the regulation of total body calcium homeostasis. As vitamin D has been implicated in the regulation of immune cells, such as T and B lymphocytes, macrophages and dendritic cells[1–3], we considered the possibility of a role for vitamin D in the mediation of inflammatory diseases including asthma.

Several studies have found that circulating 25-hydroxyvitamin D (25-(OH)D) levels are associated with risk for developing childhood asthma[2,3], wheezing[4–6]asthma severity and asthma control[7–11]. Maternal vitamin D intake during pregnancy was found to be inversely related to asthma symptoms in early childhood[12,13]. Higher 25-(OH)D levels have been associated with significantly reduced odds of hospitalizations for asthma, while low serum

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levels of this metabolite were associated with increased rates of hospitalization for asthma, increased use of controller medications and increased airway responsiveness.[9]. African American adolescents with asthma had significantly lower serum 25-(OH)D levels compared to control subjects without asthma[14]. In contrast to these findings, there have also been reports of lack of a relation between 25-(OH)D levels and asthma,[5,15–17] raising the question whether factors in the vitamin D metabolism pathway other than metabolite level may be a determining factor for asthma risk.

Vitamin D deficiency, as indicated by levels of 25-(OH)D less than 20 ng/ml (50 nmol/L), is not infrequent in the general population[18] or in children[19]. Known risk factors for vitamin D deficiency include lack of sunlight exposure, non-white ethnicity and increased skin pigmentation, obesity, and indoor confinement[14]. However 25-(OH)D levels may be influenced by other unidentified factors, raising the distinct consideration of genetic influences on circulating levels. Candidate genes that have been identified as potential determinants of circulating 25-(OH)D in GWAS studies include *VDR* (encoding the vitamin D receptor), *CYP2R1* (encoding the microsomal 25-hydroxylase), and *GC*, encoding the vitamin D binding protein (DBP), which binds circulating vitamin D metabolites[20]. *GC* is a highly polymorphic gene located at 4q11–13[21]. Many variants of DBP have been characterized by isoelectric focusing[22], but attention has increasingly centered on the two most common genetic variants—D432E (rs.7041 – c.1296TNG) and T436K (rs.4588 – c. 1307CNA). These single nucleotide polymorphisms (SNPs) in the coding region of exon 11 of *GC* encode the electrophoretically distinguishable proteins Gc1F/Gc1S and Gc2, respectively. Both variants show ethnic-specific allele frequencies based on large population studies[23], and have been shown to correlate with vitamin D metabolite levels[24–26]. More recently we have shown that the T436K variant in DBP is an important determinant of 25-(OH)D levels in healthy infants and toddlers[27]. We therefore hypothesized that specific DBP variants associated with circulating 25-(OH)D levels would be associated with increased risk for developing asthma in children.

Methods

Study Population

We accessed data from 776 healthy children who were enrolled from 2005 to 2008 (aged 6–36 months) in a study examining determinants of circulating vitamin D metabolite levels. At enrollment the subjects were healthy, and were specifically free from diseases or conditions that may affect overall nutritional status or bone metabolism. Children with a history of disorders that affected vitamin D or mineral metabolism, or who received systemic glucocorticoids, medium dose (352 mcg fluticasone or equivalent)[28] or higher of inhaled corticosteroids for age up to 4 years and those who had current or recent (within 1 month) use of anticonvulsants or other medications known to affect bone and mineral homeostasis were excluded from enrollment in the original study. The children received primary care services at one of 4 community based primary care centers in New Haven, CT. The ethnicity of the subjects was predominantly Hispanic, but included Black and Caucasian children. The study was approved by the Yale University Institutional Review Board for clinical investigation.

Study Design

This retrospective medical record review was performed from 2010 to 2011, and included demographic data, as well as detailed clinical information as it relates to development of asthma, asthma symptoms and atopic disease. Evidence for asthma as well as confirmation of an asthma diagnosis that was present in the record was based on the NHLBI EPR-3 Guidelines[28]. This included identifying symptom frequency and pulmonary function testing (impairment domain), exacerbations (risk domain), triggers, evidence of atopy, and family history. Subjects were excluded if they had developed any chronic respiratory or non respiratory disease other than asthma, had a history of prematurity < 32 weeks gestational age, liver disease such as hepatitis, renal/urologic disease (e.g., recurrent urinary tract infection), or used pharmacologic or prescription-level dosages of vitamin D or its metabolites.

Biochemical analysis

At enrollment *GC* genotype, DBP concentration and circulating levels of 25-(OH)D were determined. Serum 25-(OH)D was measured by radioimmunoassay kit methodology (DiaSorin, Stillwater, MN). Plasma concentration of DBP was measured by immunonephelometry, using a Behring Nephelometer (Behring Diagnostics, Westwood, MA), as described by Wians et al[29].

Genotype analysis

The p.D432E (rs7041) and p.T436K (rs4588) GC SNPs were genotyped, with phase assignment based on allele-specific amplification of the p.T436K site, followed by restriction endonuclease digestion of the p.D432E site, as previously described[27]. Allele-specific amplification was carried out in a 20-μL reaction mixture containing 1× PCR buffer (Qiagen, Toronto, Canada), 0.2 mM each of the four deoxynucleoside triphosphates (dATP, dGTP, dTTP, and dCTP), 50 ng genomic DNA, 0.5 U HotStarTaq (Qiagen), and 0.3–1 μM of the following primers: 5′-GGCATGTTTCACTTTCTGATCTC-3′ (forward), 5′-ACCAGCTTTGCCAGTACCG-3′ (wild-type reverse), and 5′-GCAAAGTCTGAGTGCTTGTTATGCAGCTTTGCCAGTTGCT-3′ (mutant reverse). The underlined bases in the primer sequences are mismatched nucleotides introduced to avoid cross-priming. After the initial DNA denaturation and HotStarTaq activation at 95°C for 15 minutes, the amplification went through 35 cycles of denaturation at 94°C for 20 seconds, annealing at 58°C for 20 seconds, and extension at 72°C for 20 seconds with an increment of 1 second after each subsequent cycle and a final extension at 72°C for 5 minutes at the finish. Eight microliters of the amplified products were run in a 2% NuSieve gel containing ethidium bromide and then visualized using UV illumination. The p.T436K wild-type allele produced a 246–base pair (bp) band and the mutant allele a 270-bp band. Another 8 μL of amplicon was digested with HaeIII (New England BioLabs) at 37°C overnight and the digested products were analyzed by repeat electrophoresis in 2% NuSieve gel. The DNA bands containing the wild-type p.D432E allele remained unchanged whereas those with p.D432E mutant allele were cut, producing 221-bp fragments.

Given the absence of any recombinants between the two polymorphic loci, assignment of a diplotype for each subject, based on the three haplotype alleles—wild-type (electrophoretic

variant 1f), mutant 432E (electrophoretic variant 1s), and mutant 436K (electrophoretic variant 2)—was unambiguous.

Data Analysis

Chi-square and t-tests were conducted to compare the categorical or continuous characteristics between asthmatics and non-asthmatics accordingly. Hardy-Weinberg equilibrium was tested within asthmatics, non-asthmatics and the entire population using exact test method. If significant disequilibrium existed in non-asthmatics for a certain SNP, this marker was excluded from further analysis. Otherwise, haplotype was constructed according to genotyping results. For haplotype of D432E/T436K, the reconstruction was completed directly since there was no existence of haplotype EK. The haplotypes of VDR (vitamin D receptor) and CYP2R1 (encoding the vitamin D-25 hydroxylase that catalyzes synthesis of 25-(OH)D from native vitamin D) were reconstructed using the expectation-maximization (EM) algorithm in the Proc Haplotype procedure in SAS genetics. SNPs for these 2 genes were selected based on previous studies that have shown a relationship between them and 25-(OH)D levels. Five VDR SNPs were examined: Fok1 (rs2228570), Taq1 (rs731236), Bsm1 (rs1544410), Apa1 (rs7975232) and Cdx-2 (rs11568820)[30,31]. For CYP2R1 the rs10741657 SNP was examined as it had been previously identified as associated with 25-(OH)D levels[32–34]. Multivariate logistical regression was performed for asthma outcome using haplotype, 25-(OH)D and obesity. A backward model selection strategy was used. Only variables with the p value of estimate < 0.2 were kept in the final model. The genotype effect on age, age at enrollment, DBP concentration and the circulating levels of 25-(OH)D at enrollment were examined using ANOVA method. All the statistical analyses were performed using SAS 9.2 (SAS, Cary, NC). Significance level of p-value was at 0.05, two-sided.

Results

Of the original sample of 776 subjects, 69 were excluded because of inadequate genotype information. Sufficient information to confirm or exclude the diagnosis of asthma using the NHLBI EPR3 Guidelines was available for 601 subjects. Subjects were initially divided into 2 groups by age: < 5 years and ≥ 5 years, because of the uncertainty regarding whether a diagnosis of asthma could be established in the younger children with the information available in the medical record. In 23 of the 161 younger children the diagnosis of asthma could be confirmed. We therefore included these children in the analysis of the older age group, resulting in a total of 463 subjects. The age of the youngest subjects included in this analysis was 4 years.

The characteristics of the subjects with and without asthma are shown in Table 1. There was no difference in gender distribution, age at initial enrollment, current age or obesity between the 2 groups. The prevalence of asthma was less frequent among Hispanic subjects (87 vs. 247) as compared to other ethnicities.. Because of the predominance of subjects of Hispanic ethnicity (72.1% of the total) we focused our analysis on this ethnic group to minimize any potential confounding effects of ethnicity. Therefore in the final analysis we included the 334 Hispanic children only.

As shown in Table 2, the diagnosis of asthma was confirmed in 87 of the 334 examined subjects (26.0%). There was no difference between the asthma and no asthma groups with respect to current age or age at enrollment, gender or in the prevalence of obesity. The majority of subjects with asthma were classified as having intermittent asthma (58.3%). More subjects with asthma had evidence of atopy and wheezing with viral infection compared to the group without asthma (Table 2). We did not find a difference between the groups regarding circulating levels of DBP or 25-(OH)D at enrollment.

To confirm our previous finding of the relation between genotype and vitamin D[27] we examined the relationship between *GC* genotype, circulating levels of DBP, and circulating levels of 25-(OH)D (Table 3). We confirmed that circulating 25-(OH)D was affected by the SNP encoding the DBP T436K variant in an additive manner (TT > TK > KK, P=0.019) and was not affected by D432E variance in this subset of Hispanic children (P=0.76). Circulating DBP was also affected by T436K genotype (TT > TK > KK, P <0.0001), and by the D432E variants in this group of subjects as well (EE > DE > DD, p=0.02).

We then examined the odds of developing asthma as related to *GC* haplotype, (Table 4). We found that the haplotype associated with variance at the D432E position is associated with the development of asthma. In particular the E allele appeared to be protective in this regard; i.e., subjects with haplotypes containing the E variant had lower odds of developing disease compared to those containing the D432 allele. Moreover, the effect was additive such that the proportion of children with asthma decreased with progressive substitution of the D allele (DD>DE>EE, p=0.05). Thus with respect to the protein, homozygous Gc1s/Gc1s DBP is associated with the lower odds of having asthma than Gc1f/Gc1f, and the heterozygous forms containing Gc1s (ET) were associated with lower odds than Gc1f/Gc1f. We did not find a significant effect of SNPs encoding T436K variants, nor did adjusting for obesity have an effect on this outcome (Table 4).

In contrast to the *GC* SNPs, there was no effect of the *VDR* or *CYP2R1* SNPs we examined with respect to the presence of asthma. For *VDR*, only Fok1, Taq1, and Bsm1 sites were examined as potential contributory variants, as distribution of Apa1 and Cdx-2 showed significant Hardy-Weinberg disequilibrium[35]. For the three *VDR* SNPs and the rs10741657 variant in *CYPR21*, no significant differences in allele frequencies were observed between asthmatics and controls, suggesting that these SNPs in vitamin D-related genes were not genetically associated with the risk of asthma in this group of subjects.

Discussion

In this study we found that the *GC* polymorphic bases giving rise to the major electrophoretic isoforms of DBP (rs4588 and rs7041) are significant determinants of asthma risk in inner-city Hispanic children. We demonstrated effects of these GC variants on the risk for asthma development, effects that were independent of circulating 25-(OH)D levels collected at the time of initial enrollment. Specifically, our data suggest that the Gc1s allele (ET) confers a protective effect with respect to the development of asthma.

The serum vitamin D binding protein (DBP), also known as GC-globulin, is a multifunctional protein known for its role in the transport of vitamin D metabolites. In addition to binding and transporting vitamin D, other described functions include activation of macrophages, augmenting the chemotactic effect of C5a for macrophages and neutrophils, and scavenging actin[36]. These latter functions have been demonstrated in the lung as well as in other organ systems. In a recent study of adults with chronic obstructive pulmonary disease (COPD) circulating DBP levels were inversely correlated with lung function, and sputum DBP was found to contribute to macrophage activation[37]. Other studies have also shown a role for DBP in COPD.[38–40]

Until recently little attention has been afforded the role of DBP in asthma, primarily because of the varying results observed in the few studies examining this issue[36,41,42]. However, new evidence for a role of DBP in asthma has now become available. For example, in adults with asthma, DBP levels were found to be elevated in bronchoalveolar lavage fluid of subjects after allergen challenge, independent of vitamin D metabolite levels[43]. These authors then demonstrated in a murine model of ovalbumin-sensitization/challenge, that treatment with an antibody to DBP reduced airway hyperreactivity and airway inflammation in a dose dependent manner, further supporting a role for DBP[43]. In another study DBP variants were found to be associated with asthma in a Han Chinese population, with the DK haplotype (Gc2) predicting increased risk for asthma compared to wild type DT (Gc1F)[44]. In our study, individuals harboring the ET haplotype showed significantly lower odds of developing asthma compared to wild type haplotype (DT) in this population of Hispanic children. Thus the protein variant, Gc1s/Gc1s (ET/ET) would be associated with the lowest odds of asthma compared to wild type Gc1F/Gc1 (DT/DT). This difference may be related to the different populations represented in these studies, i.e., Hispanic children vs. Han Chinese adults. As in our study, the genotypes associated with development of asthma were not associated with circulating 25 (OH) D levels. Also comparable to our study, there was no relationship between the development of asthma and polymorphisms examined in other sites of the vitamin D pathway (VDR and CYP2R1). Finally, we are aware of only 2 studies in children, other than our own, examining DBP effects on asthma. In one study[45] children with severe, therapy-resistant asthma had higher levels of DBP in BAL fluid than children with moderate asthma or non-affected controls. These authors identified a negative association between BAL DBP content, asthma control test, spirometry and inhaled corticosteroid use, supporting the possibility that DBP may play a role in childhood asthma. In the second study of a small group of Egyptian children[46] the authors examined the rs2282679 SNP in *GC*; they found that the G allele was more frequent in the children with asthma as compared to controls, and that disease was more severe in children who had 2 G alleles as compared to those with TT (wild type) or GT, suggesting that the wild type T allele was protective.

The mechanism by which mutations in DBP affect asthma risk cannot be discerned from our study. Acknowledging the fact that vitamin D metabolite levels that we analyzed were obtained at original enrollment, much before the subjects had evidence of asthma, we did not identify a relationship between serum levels of vitamin D metabolite and asthma suggesting that the effect of DBP on circulating vitamin D is not the operative mechanism.

However different isoforms of Gc have been shown to have different binding affinity for 25-(OH)D (Gc1 F and S >Gc2)[47], which may result in differences among genotypes in tissue availability of vitamin D[24]. Alternatively, DBP has a variety of direct effects on the immune system, including macrophage activation[21]

To determine if the effect risk for asthma was specifically related to DBP polymorphisms or to variance in SNPs at other sites along the vitamin D pathway, we examined candidate SNPs in VDR and CYP2R1, both of which have been shown to have some association with asthma[48–50]. In a study of young African-Americans certain VDR variants were associated with increased IgE levels and aeroallergen sensitization, higher nighttime asthma morbidity scores and lower baseline spirometry scores[51]. Furthermore, a homozygous variant of the CYP2R1 gene (rs10766197) was associated with increased risk of asthma, whereas a variant of the gene encoding CYP24A1 (rs2248137) [responsible for breakdown of 1,25(OH)^{2}D and 25-(OH)D] was associated with lower vitamin D levels but not risk of asthma[51]. We did not find any difference in the genotype and allele frequencies at these 2 sites, suggesting that these SNPs do not affect the risk of developing asthma in this population of Hispanic children.

In summary, we identified a DBP genotype [ET/ET (Gc1s/Gc1s)] that is protective for the development of asthma in a population of inner-city Hispanic children. Whether the protective effect of this genotype occurs in other ethnic groups will need to be further studied, in addition to elucidating the mechanism by which such protection is incurred.

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Table 1

Demographics of all subjects examined. Data are presented as N (%) for categorical variables and Mean (SD) for continuous variables.

Characteristics	Total N=463	With asthma N=138	Without asthma N=325	p value‡
Gender				0.24
Female	242 (52.4)	66 (48.2)	176 (54.2)	
Male	220 (46.6)	71 (51.8)	149 (45.9)	
Ethnicity				**0.01**
Hispanic	334 (72.1)	87 (63.0)	247 (76.0)	
Black/African American	98 (21.2)	40 (29.0)	58 (17.8)	
Caucasian/other	31 (6.7)	11 (8.0)	20 (6.2)	
Age (years)	5.8 (1.0)	5.6 (1.2)	5.9 (0.9)	0.06
Age at enrollment (years)	1.8 (0.7)	1.7 (0.8)	1.8 (0.7)	0.38
Age at diagnosis*	2.1 (1.4)	2.1 (1.4)	-	
Obese				0.07
Yes	32 (6.9)	14 (10.2)	18 (5.5)	
No	430 (93.1)	123 (89.8)	307 (94.5)	
Asthma Class				
Intermittent		78 (56.5)		
Mild persistent		26 (14.5)		
Moderate persistent		26 (14.5)		
N/A		8 (5.8)		
DBP at enrollment, (mg/L)	196.8 (27.7)	192.8 (23.8)	198.7 (29.2)	**0.04**
25-(OH)D at enrollment, (nmol/L)	65.9 (24.3)	67.8 (37.3)	65.1 (16.0)	0.43

*asthma first diagnosed per the medical record

‡Chi-square test for categorical variables and t-test for continuous variables

Table 2

Demographics, vitamin D metabolite and DBP levels at original enrollment of *Hispanic* subjects with and without asthma. Data are presented as N (%) for categorical variables and Mean (SD) for continuous variables.

Characteristics	Total N=334	With asthma N=87	Without asthma N=247	p-value
Gender				0.31
Female	177 (53.0)	42 (48.3)	135 (54.7)	
Male	157 (47.0)	45 (51.7)	112 (45.3)	
Age, current (years)	5.9 (1.0)	5.8 (1.1)	5.9 (0.9)	0.23
Age at enrollment (years)	1.7 (0.7)	1.7 (0.7)	1.8 (0.7)	0.36
Age at diagnosis*	2.1 (1.4)	2.1 (1.4)	-	
Obese				0.13
Yes	26 (7.8)	10 (11.5)	16 (6.5)	
No	308 (92.2)	77 (88.5)	231 (93.5)	
Asthma Class				
Intermittent		49 (58.3)		
Mild persistent		20 (23.8)		
Moderate persistent		15 (17.9)		
Allergic Rhinitis				<0.0001
Yes	51 (15.3)	29 (33.3)	22 (8.9)	
No	282 (84.7)	58 (66.7)	224 (91.1)	
Allergies				0.01
Food allergy	122 (36.5)	28 (32.2)	94 (38.1)	
Medical allergy	26 (7.8)	13 (14.9)	13 (5.3)	
No	186 (55.7)	46 (52.9)	141 (56.7)	
Eczema				0.046
Yes	113 (33.8)	37 (42.5)	76 (30.8)	
No	221 (66.2)	50 (57.5)	171 (69.2)	
Wheezing with viral infections				<0.0001
Yes		82 (94.3)	43 (17.6)	
No	207 (62.4)	5 (5.8)	202 (82.5)	
DBP at enrollment (mg/L)	198.8 (26.5)	194.1 (24.6)	200.5 (27.0)	0.08
25-(OH)D at enrollment (nmol/L)	67.7 (26.4)	72.3 (45.7)	66.2 (14.7)	0.23

* asthma first diagnosed per the medical record

Table 3

Demographics, DBP and 25-(OH) D level by genotype in Hispanic subjects. T436K (upper panel) and D432E (lower panel)

	TT (N=207)	TK (N=113)	KK (N=14)	p-value#
Age (years)	5.9 (1.0)	5.9 (0.9)	6.1 (0.9)	0.60
Age at enrollment (years)	1.8 (0.7)	1.7 (0.7)	2.0 (0.7)	0.25
DBP	204.3 (26.4)	189.7 (24.0)	183.3 (23.3)	<0.0001
25-(OH)D at enrollment	70.9 (30.9)	62.1 (15.1)	65.1 (13.1)	0.02

	DD (N=111)	DE (N=176)	EE (N=47)	p-value#
Age (years)	5.9 (1.0)	5.9 (0.9)	5.9 (1.0)	0.98
Age at enrollment (years)	1.7 (0.7)	1.8 (0.7)	1.7 (0.8)	0.99
DBP	194.3 (27.8)	198.9 (24.9)	203.8 (27.5)	0.02
25-(OH)D at enrollment	67.2 (41.4)	67.3 (14.2)	70.4 (13.7)	0.76

ANOVA comparing continuous variables

Table 4

Comparing odds of having asthma between variants in DBP in Hispanic subjects.

			Crude		Adjusting for Obesity	
	Total number with haplotype	**Number (%) with asthma**	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p -value**
D432E				0.03		0.02
ET/DT (Gc1S/1F)	115	30 (26.1)	0.63 (0.41, 0.95)		0.62 (0.41, 0.94)	
ET/ET (Gc1S/1S)	47	8 (17.0)	0.39 (0.17, 0.90)		0.38 (0.17, 0.88)	
T436K				0.30		0.27
DK/DT (Gc2/1F)	52	16 (30.8)	0.78 (0.49, 1.24)		0.77 (0.48, 1.23)	
DK/DK (Gc2/2)	14	3 (21.4)	0.61 (0.24, 1.55)		0.59 (0.23, 1.51)	
Double heterozygote				0.06		0.049
DK/ET (Gc2/1S)	61	14 (23.0)	0.49 (0.23, 1.02)		0.48 (0.23, 0.99)	
Reference				-		-
DT/DT (Gc1F/Gc1F)	45	16 (35.6)	1.00		1.00	

OR: odds ratio, CI: confidence interval.