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Vitamin D Binding Protein Haplotype is Associated with Hospitalization for RSV Bronchiolitis

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Abstract

Background—Between 75,000–125,000 U.S. infants are hospitalized for respiratory syncytial virus (RSV) bronchiolitis each year. Up to half will be diagnosed with asthma in later childhood. Vitamin D deficiency has been associated with susceptibility to asthma and respiratory infections. Measured vitamin D is largely bound to vitamin D binding protein (VDBP); VDBP levels are influenced by its gene (GC) haplotype.

Objective—We assessed the relationship between polymorphisms rs7041 and rs4588, which define haplotypes GC1s, GC1f, and GC2, and RSV bronchiolitis susceptibility and subsequent asthma.

Methods—We retrospectively recruited 198 otherwise healthy children (93% White) hospitalized for severe RSV bronchiolitis in Boston and 333 parents into a follow up study to assess asthma diagnosis. Data were analyzed using family-based genetic association tests. We independently validated our results in 465 White children hospitalized with RSV bronchiolitis and 930 White population controls from the Netherlands.

Results—The rs7041_C allele (denoting haplotype GC1s) was overtransmitted (P=0.02, additive model) in the entire Boston cohort, and in Whites (P=0.03), and in those subsequently diagnosed with asthma (P=0.006). The GC1f haplotype was undertransmitted in the White and asthma subgroups (both P=0.05). The rs7041_C allele was also more frequent in the RSV bronchiolitis group compared to controls (OR 1.12, 95% CI 1.02, 1.4, P=0.03) in the Netherlands; especially in mechanically ventilated patients (P=0.009).

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Conclusion and Clinical Relevance—GC1s haplotype carriage may increase the risk of RSV bronchiolitis in infancy and subsequent asthma development. The GC1s haplotype is associated with higher VDBP levels, resulting in less freely-available vitamin D.

Keywords

RSV; bronchiolitis; asthma; children; infants; polymorphisms; vitamin D binding protein; haplotype; GC; vitamin D

INTRODUCTION

Respiratory Syncytial Virus (RSV) bronchiolitis, the most commonly reported infection in infancy, leads to hospitalization in up to 2.5% of children in their first 6 months of life.[1,2] Approximately 40% of previously healthy infants hospitalized for RSV bronchiolitis will later be diagnosed with asthma.[3–5] We have recently shown that RSV bronchiolitis is causatively related to subsequent recurrent wheeze in otherwise healthy preterm infants.[6] Mounting evidence from animal and human studies supports a genetic basis for susceptibility to severe infections [7–12] and there is increasing evidence that host genetics influence susceptibility to RSV bronchiolitis in infants.[13]

Vitamin D, a steroid hormone synthesized in the skin via exposure to sunlight, is essential for innate immunity and influences lung function in asthmatics.[14,15] The activated form of vitamin D (1,25-dihydroxyvitamin D or 1,25(OH)D) is an immune modulator[16, 17] influencing Th1, Th2 and Th17 inflammatory processes and T-cell regulatory cell function.[18,19] Respiratory epithelial cells convert 25(OH)D to its active form[20], with potent antiviral effects.[21] We recently reported that vitamin D deficiency in healthy neonates is associated with an increased risk of RSV lower respiratory tract infections in the first year of life.[22] In children and adults, increased respiratory infections and asthma exacerbations have also been associated with vitamin D deficiency [23,24] and with polymorphisms in the vitamin D receptor gene.[25,26] The above evidence supports a role for genes in the vitamin D pathway in RSV bronchiolitis susceptibility.

The majority of vitamin D circulates bound to vitamin D binding protein (VDBP), which is also known as Gc-globulin. Variants rs7041 and rs4588 in the VDBP gene (GC) result in three major haplotypes commonly denoted GC1f, GC1s and GC2. GC haplotype carriage is associated with the amount of VDBP measured in circulating blood, and with VDBP binding affinity.[27,28] Therefore, for the same measured level of 25(OH)D, the level of freely available (unbound) vitamin D will vary depending on VDBP haplotype carriage. Using a cohort of previously healthy infants hospitalized in Boston, we used a family-based approach to evaluate the association between the two VDBP haplotype tagging gene variants and the risk of hospitalization for RSV bronchiolitis and the subsequent development of asthma. We tested our findings for confirmation in an independent cohort from the Netherlands of previously healthy infants hospitalized for RSV bronchiolitis.

METHODS

Study design and subjects

We retrospectively recruited otherwise healthy children hospitalized between January 1990 and September 2010 for RSV bronchiolitis at Boston Children's Hospital. Eligible children were identified retrospectively through the hospital database of positive RSV laboratory test results (rapid test and/or culture). The follow-up study began in April of 2000, and all eligible children hospitalized prior to that up to January 1990 were sent a recruitment letter. Children hospitalized from April 2000-September 2010 were sent an initial follow-up survey between 6 months to 1 year after their hospitalization. The study was approved by Boston Children's Hospital's Institutional Review Board.

Diagnosis of bronchiolitis was based on coryzal illness, with or without fever, in children with documented respiratory symptoms or signs that included any of the following: 1. Tachypnea, retractions and other signs of increased work of breathing; 2. Wheezing; 3. Need for supplementary FiO2 to maintain SpO2 >95%; or 4. Hyperinflation and/or patchy infiltrates on chest radiograph. Children were excluded if they were ≥2 years of age at hospital admission, born prematurely at <36 weeks of age, or diagnosed with a comorbid condition that would predispose them to RSV bronchiolitis, including but not limited to the following: cardiac disease, multiple congenital anomalies inherited or secondary immune defects causing immunosuppression, and chronic pulmonary or upper airway disease.

Families of 805 potentially eligible patients were approached by mail for participation in a follow-up study to determine the development of subsequent asthma. There were 221 families that had relocated and could not be reached (27.4%), 307 who did not respond to 3 mailings, and 277 that agreed to participate (34.4%). We were not able to extract data from the charts of the patients that did not consent. After further review, 198 (24.6%) were confirmed eligible and included. Race and ethnicity were determined by parental report. Families were sent a follow-up survey every 6–12 months. Subsequent asthma was defined as any report by the child's parent or caregiver of a physician diagnosing the child with asthma at any follow-up point.

We tested positive findings for confirmation using a case-control approach in an independent cohort of 465 infants with no prior history of respiratory conditions or wheeze who were hospitalized for RSV bronchiolitis in the Netherlands and 930 population controls. The details of these cohorts have been described previously.[29,30] Almost all infants (97.9%) were hospitalized in the first 12 months of life. Only native White Dutch children (defined as being the third generation born in the Netherlands) were used for analysis. The comparison group of native Dutch controls was randomly taken from a large Dutch health exam survey population called the Regenboog study, and their recruitment and follow-up has previously been reported in detail.[29,31]

Laboratory and Genotyping Methods

Saliva samples from the proband and consenting biologic parents were collected using Oragene saliva kits (DNA Genotek Inc., Ontario, Canada), with DNA extracted according to the manufacturer's protocol. We analyzed genotyping results from 137 complete family trios

and 59 parent-child duos. Blood was collected from 22 children and DNA was extracted using the Gentra Puregene Blood Kit (QIAGEN). DNA concentration and quality was quantified using Quant-it™ DNA Assay Kits (Life Technologies).

We genotyped rs4588 and rs7041 using the Sequenom mass spectrometry genotyping platform. Secondary modified single-primer minisequencing reactions were formed in multiplex and were analyzed using the Bi-flex MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA, USA). Spectral output was analyzed using SpectroTYPER-RT software (Sequenom Inc, San Diego, CA, USA). As a quality control measure, genotyping was repeated for at least 8% of the sample for each SNP and was tested for discordance. For the cohort from the Netherlands genotyping was performed by K-Bioscience (United Kingdom) using KASP technology.[29]

Statistical Methods

Analyses of the Boston cohort (entire cohort and then Whites only) were conducted using R (version 2.15.2 on Windows platform) and FBAT (version 2.0.4 on Linux64 platform).[32,33] We tested different genetic models (additive, dominant and recessive) for linkage and association between the polymorphisms using the transmission disequilibrium test (TDT) in FBAT, and using the HBAT function for haplotypes. Associations were performed for two major phenotypes 1.) RSV bronchiolitis susceptibility; and 2.) Asthma reported subsequent to RSV bronchiolitis.[34,35] We performed the analysis in the entire cohort (all races and ethnicities) and in Whites only.

For the cohort from the Netherlands, we used SPSS (version 20) to compare the frequencies of the alleles, genotypes and haploptyes in the children hospitalized with RSV bronchiolitis to the population controls with Chi Squared tests. Children who were mechanically ventilated during their admission were secondarily compared as a subgroup to the population controls.

RESULTS

The Boston RSV bronchiolitis cohort (n=198) was mostly male (61%), and predominantly white (93%). The median age at hospital admission for bronchiolitis was 54 days (IQR 31, 126 days). The median age at last follow-up was 9.2 years (IQR 4.5, 13.4 years). There were 71 children (36%) who were diagnosed with asthma at some time point after their hospitalization, and 85 children (43%) who did not receive a doctor’s diagnosis of asthma by 4 years of age. There were 42 children (22%) who did not receive a doctor’s diagnosis of asthma, but who were all less than 4 years of age at their last recorded follow up visit (median age 0.9 years (IQR 0.25, 2.25 years), and we categorized their asthma status as “indeterminate”. Table 1 shows the characteristics of the patients.

There were no Mendelian errors detected in the dataset. After excluding 2 probands where genotyping failed, we used FBAT to analyze 196 pedigrees (146 nuclear families, 531 individuals). Restricting the analysis to Whites resulted in 171 pedigrees (103 nuclear families, 438 persons). The associations at the allele level are reported in Table 2. We found overtransmission of the rs7041_C allele in children with RSV bronchiolitis (P=0.02), in

Whites (P=0.03) and in the subgroup reporting a subsequent asthma diagnosis (P=0.006 overall, P=0.007 in Whites). The rs7041_C allele denotes the GC1s VDBP haplotype, therefore the association was similar at the haplotype level. The 7041_A allele is present in two VDBP haplotypes (GC1f and 2). As shown in Table 3, there was under-transmission of the GC1f haplotype in Whites and in the asthma subgroup (both p <0.05). Sensitivity analysis showed that our findings did not change if we classified the children with "indeterminate" asthma status at follow up as having a doctor's diagnosis of asthma or no asthma diagnosis. Results were similar when dominant or recessive statistical models were applied.

The results of our analysis in the cohort of native white children from the Netherlands are reported in Table 4. Allele frequencies were similar to that reported in Whites in the Boston cohort. The rs7041_C allele was more frequent in infants hospitalized for RSV bronchiolitis than in controls (OR 1.12, 95% CI 1.02, 1.4, P=0.03), especially in mechanically ventilated patients (OR 1.57, 95% CI 1.12, 2.22, P=0.009). This corresponded to an increased frequency of the GC1s haplotype and a decreased frequency of the GC1f haplotype as was seen in the Boston RSV bronchiolitis cohort.

DISCUSSION

Using a family-based study design, we identified a higher than expected rate of transmission of the GC1s VDBP haplotype from heterozygous parents to their infants who were hospitalized for RSV bronchiolitis in Boston. We confirmed this finding in an independent cohort of infants hospitalized for RSV bronchiolitis from the Netherlands using population controls. The association became stronger in the subgroup of children from Boston who were identified on follow-up to have a clinical diagnosis of asthma. It was also stronger in the subgroup of children from the Netherlands who were mechanically ventilated for RSV bronchiolitis. Both cohorts were similar, comprised mostly of White children and excluding infants with comorbid conditions that would predispose them to more severe illness with RSV infection.

The VDBP gene GC is a likely candidate for genetic susceptibility to RSV bronchiolitis. Apart from being the main carrier of Vitamin D throughout the body, VDBP has several functions related to immune response. VDBP plays major functional roles in the lung including clearance of G-actin, and has immunomodulatory functions which vary according to GC haplotype.[28,36] Multiple studies show that GC2 carriage is associated with lower VDBP levels compared to carriage of GC1 (GC1S or GC1f).[36] Gc1, but not Gc2, protein isoforms are transformed by glycohydrolysis into Gc-MAF, a very potent macrophage activating factor that could influence acute and chronic lung inflammation. Vitamin D deficiency has been associated with an increased risk of respiratory infections and asthma.[37] GC2 and GC1s polymorphisms have been associated with lower vitamin D levels in four genome-wide association studies.[38–40] The GC2 haplotype has been associated with asthma in adults.[41] The GC2, GC1f and GC1s haplotypes have been associated with increased and lowered risk in COPD patients; no study has shown carriers of GC1s to have an increased risk.[36,42,43] GC2 haplotypes have been associated with increased susceptibility to tuberculosis and rheumatic fever.[36] GC2 haplotype carriers have a greater

response to vitamin D supplementation.[44] Our cohort was made up mostly of individuals of European ancestry who carry mainly the GC1s or GC2 haplotype; GC1f is more common in individuals with African ancestry.[36] Mathematical models estimate that GC1s haplotype carriers to have much less freely available vitamin D compared to GC2 carriers based on VDBP levels and VDBP binding affinity for 25(OH)D [45,46].

Reports of associations between RSV bronchiolitis susceptibility, post-infection outcome and innate immunity genes have been published on patient cohorts from nine countries including Germany [47], Finland [48], England [49], The Netherlands [50], Israel [51], Greece [52], Korea [53], China [54] and South-Africa.[26] Prior reports suffer from small sample size and lack of independent replication of positive findings with the exception of Kresfelder et al.[26] who confirmed the association reported by Janssen, Bont and colleagues [29] between a vitamin D receptor polymorphism and the risk of hospitalization for RSV bronchiolitis. A major strength of our study is that our RSV bronchiolitis cohorts were some of the largest reported to date, and findings were in both cohorts.

Our study has several limitations. In the Boston cohort, patients were identified retrospectively after hospitalization. We cannot rule out the possibility of selection bias because we did were not allowed to extract data from the charts of individuals without consent to determine if they differed from those who enrolled. We used parental report of a doctor's diagnosis of asthma instead of clinical records which may be subject to recall bias. In some patients, we could not determine if they had developed asthma because their last follow-up occurred before the age of 4 years. However, sensitivity analysis showed that categorizing this indeterminate group as asthma or no asthma did not influence our results. We were unable to confirm in the Netherlands cohort that the association between GC1s was strongest in those patients who later developed asthma because patients were not followed up to determine asthma status. Although infants mechanically ventilated for RSV bronchiolitis were the most likely to carry the GC1s haplotype in the Netherlands, there were too few infants requiring mechanical ventilation in the Boston cohort to confirm this finding. Although an association between low cord blood vitamin D levels and RSV bronchiolitis in infancy has been reported in another cohort in the Netherlands [22], we did not have blood samples at birth or during follow up in a sufficient number of patients in either cohort to assess the role of levels of VDBP or vitamin D in our cohorts.

We report the association of a functional VDBP gene variant and severe RSV bronchiolitis in early life in a family based study and its replication in a case-control study in a second cohort. We speculate that either due to the influence of the GC1s haplotype on higher levels of VDBP and thus lower levels of unbound freely available vitamin [36,55,56], or due to its activation of macrophages in the lung via GcMAF [36], carriage of this haplotype may lead to susceptibility to RSV bronchiolitis and more severe respiratory complications. Our findings suggest that future study of the role of vitamin D in susceptibility to RSV bronchiolitis needs to include levels of VDBP and GC haplotypes as well as vitamin D levels to further understand the role of the vitamin D pathway in RSV bronchiolitis.

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ABBREVIATIONS

RSV	Respiratory Syncytial Virus
LRTI	lower respiratory tract infection
SNP	single nucleotide polymorphism
VDBP	Vitamin D Binding Protein
TDT	Transmission disequilibrium test
FBAT	Family-based association test

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KEY MESSAGES

Vitamin D binding protein (VDBP) haplotypes influence free vitamin D levels. We report an association between a VDBP haplotype and hospitalization for RSV bronchiolitis in infancy in two independent cohorts.

Table 1

Characteristics of the infants hospitalized with RSV bronchiolitis in Boston and in the Netherlands.

Characteristics	Boston N = 198 (%)	Netherlands N = 465
Gender		
Male	121 (61%)	265 (57%)
Female	77 (39%)	200 (43%)
Race		
White non-Hispanic	179 (90%)	465 (100)
White Hispanic	6 (3%)	
Other	13 (7%)	
	Median (IQR)	**Median (IQR)**
Age on Hospital Admission (days)	54.0 (30.8, 126)	63.5 (37, 130)
Age at Last Follow-up (years)	9.2 (4.5, 13.4)	
Level of Respiratory Support	**N (%)**	**N (%)**
During Hospitalization		
None	64 (32.5%)	
Supplemental oxygen	103 (52.3%)	
Non-invasive ventilator support *	10 (5.1%)	
No invasive ventilator support *	178 (89.9%)	385 (83%)
Intubated and on ventilator	20 (10.1%)	80 (17%)
Doctor's Diagnosis of Asthma		
Confirmed	71 (35.9)	NA
None	85 (42.9)	NA
Unclear **	42 (21.2)	NA

IQR = interquartile range; NA = not applicable

* High flow nasal cannula FiO2, CPAP or BiPAP; No invasive ventilator support is a sum of the categories above it.

** Patient had no documented doctor's diagnosis of asthma but were all < 4 years of age on their last follow-up assessment so final asthma status could not be determined.

Table 2

Association at the allele level between two polymorphisms in the vitamin D binding protein in children hospitalized for RSV bronchiolitis in Boston using a family-based approach (FBAT, additive model).

	SNP	Allele Frequency (%)	Allele	Informative Trios (N)	Z	p
RSV Bronchiolitis						
All Races	rs4588	25.7	T	88	−0.847	0.40
		74.3	G		0.847	
	rs7041	56.4	C	106	2.258	**0.02** *
		43.6	A		−2.258	
White only	rs4588	25.8	T	81	−0.583	0.56
		74.2	G		0.583	
	rs7041	58.9	C	101	2.213	**0.03** **
		41.1	A		−2.213	
Subsequent Asthma						
All Races	rs4588	25.7	T	31	−1.000	0.32
		74.3	G		1.000	
	rs7041	56.4	C	38	2.771	**0.006**
		43.6	A		−2.771	
White Only	rs4588	25.8	T	28	−0.870	0.38
		74.2	G		0.870	
	rs7041	58.9	C	35	2.714	**0.007**
		41.1	A		−2.714	

VDBP = Vitamin D Binding Protein; SNP = Single Nucleotide Polymorphism; RSV = Respiratory syncytial virus

* dominant model P=0.007;

** dominant model P=0.004

Table 3

Association at the haplotype level between two polymorphisms in the vitamin D binding protein in children hospitalized for RSV bronchiolitis in Boston using a family-based approach (FBAT, additive model).

	Haplotype frequency (%)	Haplotype	Informative Trios (N)	Z	p
RSV Bronchiolitis					
All Races	55.8	GC (1s)	93	2.269	**0.02**
	25.7	TA (2)	74	−1.260	0.21
	18.1	GA (1f)	69	−1.647	0.10
	0.004	TC	13	1.025	0.31
White only	57.8	GC (1s)	88	2.225	**0.03**
	25.7	TA (2)	67	−0.985	0.32
	16.0	GA (1f)	64	−1.939	**0.05** *
	0.004	TC	13	1.026	0.3
Subsequent Asthma					
All Races	55.8	GC (1s)	36	2.596	**0.009**
	25.7	TA (2)	29	−1.371	0.17
	18.1	GA (1f)	24	−1.985	**0.047**
White only	57.8	GC (1s)	33	2.534	**0.01**
	25.7	TA (2)	26	−1.256	0.21
	16.0	GA (1f)	22	−2.054	**0.04**

VDBP = Vitamin D Binding Protein; SNP = Single Nucleotide Polymorphism; RSV = Respiratory syncytial virus

* P=0.02 recessive model

Table 4

VDPB related SNPs in a cohort of native white Dutch children hospitalized for RSV bronchiolitis; association at allele and haplotype (rs4588/rs7041) level

		Allele Frequency						**Haplotype Frequency**		
SNP		***RSV Bronchiolitis N (%)***	***Controls N (%)***	***OR***	***[95% CI]***	***p***		**RSV Bronchiolitis N (%)**	**Controls N (%)**	**p**
All										
rs4588	G	665 (72.9)	1320 (73.2)	1.01	[0.85–1.21]	0.89	GC (1s)	535 (59.4)	992 (55.4)	**0.01**
	T	247 (27.1)	484 (26.8)				TA (2)	245 (27.2)	480 (26.8)	
							GA (1f)	120 (13.3)	318 (17.8)	
rs7041	A	375 (40.8)	834 (45.2)	1.2	[1.02–1.40]	**0.03**				
	C	543 (59.2)	1010 (54.8)							
Mechanically ventilated										
rs7041	A	53 (34.4)	834 (45.2)	1.57	[1.12–2.22]	**0.009**	GC (1s)	101 (65.6)	992 (55.4)	**0.01**
	C	101 (65.6)	1010 (54.8)				TA (2)	39 (25.3)	480 (26.8)	
							GA (1f)	14 (9.1)	318 (17.8)	

VDBP = Vitamin D Binding Protein; SNP = Single Nucleotide Polymorphism; RSV = Respiratory syncytial virus