Human Immunology 74 (2013) 234–240

Contents lists available at SciVerse ScienceDirect

journal homepage: www.elsevier.com/locate/humimm

Association of vitamin D receptor gene polymorphisms with susceptibility to asthma in Tunisian children: A case control study

Haifa Maalmi [a,*], Fayçal Haj Sassi [a], Anissa Berraies [a,b], Jamel Ammar [a,b], Kamel Hamzaoui [a], Agnes Hamzaoui [a,b]

[a] Université de Tunis El Manar, Faculté de médecine de Tunis, 99/08-40 homeostasis and cell dysfunction unit research, 15 Rue Djebel Lakdar 1007, Tunis Tunisia
[b] Hopital Abderrahmane MAMI, Service de Pneumologie et Université de Tunis El Manar, Tunisia

ARTICLE INFO

Article history:
Received 10 May 2012
Accepted 15 November 2012
Available online 29 November 2012

ABSTRACT

Background: Vitamin D and its nuclear receptor (VDR) are linked to asthma in a genetic and immunologic basis. Polymorphisms in the VDR gene may alter the actions of vitamin D and then influence the development and the severity of asthma.
Aims: We aimed at elucidating the genetic association of VDR gene polymorphisms with susceptibility to asthma in Tunisian children and with serum vitamin D levels.
Methods: The study included 155 patients recruited from Abderrahmen MAMI hospital in Tunisia and two hundred twenty five healthy individuals matched with patients in age and sex for comparison. VDR genotypes were determined by PCR-RFLP method using endonuclease *FokI*, *BsmI*, *TaqI* and *ApaI* and vitamin D was assessed with a radioimmunoassay kit.
Results: The distribution of genotype frequencies differed significantly between asthmatics and controls (*FokI*: $P = 0.04$; *BsmI*: $P = 0.006$; *TaqI*: $P = 0.006$). Haplotype analyses revealed a significant association between bAt and bat haplotypes and asthma ($P = 0.00076$, $P = 0.016$). When patients were stratified according to atopic status and stage of severity, no significant association was detected with VDR variants. No association was found between VDR SNPs and serum 25-hydroxyvitamin D levels.
Conclusion: Our study shows a relation between VDR gene polymorphisms and susceptibility to asthma in children.

1. Introduction

Asthma is the most common disease of childhood and is in large part attributable to genetic factors [1]. Airway inflammation is the main characteristic of this chronic disease. Dysregulated Th cells and abnormal inflammatory response, including increased levels of IL-4, IL-5, and IL-13 may be responsible of the previous cited inflammation status [2]. The result of this exacerbated response is followed with eosinophils, lymphocytes and macrophages infiltration of mucosal airways tissue [2].

In the last years, vitamin D and its nuclear receptor VDR have emerged as new factors contributing to the pathogenesis of asthma. Vitamin D has potent immunomodulatory properties, on cells of the innate and adaptive immune system which may modulate the airway inflammation. $1,25(OH)_2D$ stimulates innate immune responses through facilitating production of antimicrobial proteins such as cathelicidin and by inhibiting NF-κB signaling which could result in decreased induction of NF-κB-linked chemokines and cytokines [3]. Therefore modulating chemokines production may have the potential to prevent excessive inflammation. On the other hand, $1,25(OH)_2D$ inhibits the surface expression of MHC-II-complexed antigen and of co-stimulatory molecules on APC (like DCs), in addition to production of IL-12 and IL-23 cytokines, thereby indirectly shifting the polarization of T cells from a Th1 and Th17 phenotype towards a Th2 phenotype. In addition, $1,25(OH)_2D$ directly affects T cell responses, by inhibiting the production of Th1 cytokines (IL-2 and IFN-γ), Th17 cytokines (IL-17 and IL-21), and by stimulating Th2 cytokine production (IL-4). Moreover, $1,25(OH)_2D$ favors Treg cell development via modulation of DCs and by directly targeting T cells [4].

Further studies demonstrate that asthma may be linked to vitamin D on a molecular genetic basis. Genome scans have identified linkage in several regions, including region q13–23 of chromosome

Abbreviations: VDR, vitamin D receptor; SNP, single nucleotide polymorphism.

* Corresponding author. Address: Medicine University Tunis, 15 Rue, Djebel Lakdar, 1007 Tunis, Tunisia. Fax: +216 71 569 427.
E-mail addresses: maalmi2010@gmail.com (H. Maalmi), faycalhsassi@yahoo.fr (F. Haj Sassi), anissa.berraies@hotmail.com (A. Berraies), jamelariana@gmail.com (J. Ammar), Kamel.hamzaoui@gmail.com (K. Hamzaoui), agnes.hamzaoui@gmail.com (A. Hamzaoui).

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http://dx.doi.org/10.1016/j.humimm.2012.11.005

12, housing the vitamin D receptor (VDR) gene [5–7]. A number of association studies were previously conducted in different populations and ethnic groups. Two different family-based studies in North American subjects reported an association between VDR polymorphisms and asthma [8,9]. However another research involving German subjects did not show this link [10]. The discrepancies are probably due to differences of populations origins and different outcomes measured.

This immune and genetic control of vitamin D may influence the pathogenesis of asthma. Epidemiological studies confirmed the existence of a positive link between vitamin D deficiency and asthma clinical characteristics. Lower vitamin D circulating levels are associated with an increase in asthma severity including asthma exacerbation and odds of hospitalizations [11,12], airway responsiveness [11,13,14] and inhaled steroid use [11,15].

These findings indicated that $1.25(OH)_2D$ and its specific nuclear receptor, VDR, may be closely associated with asthma risk. Nevertheless, several genetic variations (single nucleotides polymorphisms; SNP) that have been identified in the VDR gene may influence either its expression or its function which may cause alteration in the vitamin D activity. In addition, many of the VDR target genes are involved in the regulation of vitamin D metabolite concentrations via a classical endocrine feedback loop [16]. Any defect in the VDR expression or function could influence concentrations of vitamin D metabolites.

Four single nucleotide polymorphisms (SNPs) in the VDR gene have been well investigated by genetic associations studies, namely *FokI* F > f (rs2228570), *BsmI* B > b (rs1544410), *ApaI* A > a (rs7975232), and *TaqI* T > t (rs731236). Allele F of the Fok-I SNP creates an alternative ATG initiation codon in exon 2 leading to a three amino acids longer VDR protein. The *ApaI*, *BsmI* and *TaqI* polymorphisms are located near the 3′end of the VDR gene; the *BsmI* and *ApaI* SNPs are both located in intron 8, and the *TaqI* is a silent SNP in exon 9. A number of association studies were previously conducted in different populations and ethnic groups. Some of them have suggested association between one or more of these SNPs and asthma [8,9,17] but others have failed to confirm this finding [10,18].

In this study, we aimed at elucidating the genetic association of VDR polymorphisms with susceptibility to asthma in Tunisian children. We also assessed the eventual association between the VDR gene polymorphisms and serum level of 25(OH)D in asthmatics and healthy subjects.

2. Subjects and methods

2.1. Subjects

A total of 155 asthmatic children (59 girls and 96 boys) were recruited from the department of Pediatric and Respiratory Diseases, Abderrahmane MAMI Hospital of Chest Diseases, Ariana, Tunisia. The mean age of the subjects was 9.1 years. The diagnosis and classification of the clinical severity of asthma was based in clinical symptoms and lung function according to the GINA guidelines [19]. Patients were classified as intermittent and mild persistent asthma (N = 82), moderate persistent asthma ($N = 63$), and severe persistent asthma cases ($N = 10$) (Table 1).

Skin sensitivity to specific allergens was used to define the atopic status of 92 patients. We defined atopy by a positive skin test reaction characterized by a weal of at least 3 mm in diameter, to one or more allergens in the presence of a positive histamine control and a negative uncoated control. A panel of common aeroallergens was used. Seventy patients were considered atopic.

In addition, we recruited a group of 225 control children (aged 4–16 years, means 9.5), from the emergency department of Tunis Children Hospital with no respiratory nor allergy manifestations. All data and sample collections for this study were approved by Local Ethics Committee. Informed parental consent was obtained for all children.

Table 1
Clinical characteristics of the study sample.

	Asthmatics	Controls
Number of subjects	155	225
Mean age (range)	9.1 (4–16)	9.5 (2–16)
Sex ratio (girls/boys)	0.6 (59/96)	0.7 (99/126)
Total frequency of females	38%	44%
Total frequency of males	62%	56%
Atopic status ($n = 92$)		
Atopic (n, %)	70 (76%)	–
Non atopic (n, %)	22 (24%)	–
Severity		
Intermittent and mild	82	–
Moderate	63	–
Severe	10	–

2.2. VDR polymorphisms genotyping

Genomic DNA was extracted from peripheral blood leukocytes using the salting out procedure as described [20]. *BsmI*, *TaqI* and *ApaI* VDR polymorphisms were assessed in 155 patients and 225 controls and *FokI* VDR polymorphism was assessed for 155 patients and only 152 controls. All of the genotyping was done by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Primer sequences used for amplification of *FokI*, *BsmI*, *TaqI* and *ApaI* polymorphisms were as previously described and are listed in Table 2 [21].

The PCR was performed in a 50 μl reaction volume containing 2.4 μM of each primer, 10 mM of each dNTP, 1x PCR buffer, 1.5 mM $MgCl_2$, 2 U of Taq DNA polymerase (Fermentas), and 100 ng of genomic DNA, diluted to the final volume with H_2O. The running conditions were: pre-denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 94 °C, annealing temperature at 60 °C, and extension at 72 °C for each 1 min, respectively. Finally, extension was conducted at 72 °C for 5 min.

Amplified fragments were digested with the appropriate restriction enzyme (Fermentas) according to the manufacturer's instructions and visualized on a 3% agarose gel. The usual nomenclature for restriction fragment length polymorphism alleles was used in this study [21,22]. The lowercase allele represents the presence of the restriction site (f, b, a, or t) and the uppercase allele represents the absence of the restriction site (F, B, A, or T).

2.3. Serum 25(OH)D levels

The collected serum was immediately shaded from direct light and stored at −20 °C. All samples were analyzed simultaneously at the same laboratory, using a same technique conducted by one technician. Serum concentrations of 25(OH)D were measured with a radioimmunoassay kit (Dia-Sorin, Stillwater, MN, USA) [23] and values were reported in nanograms per milliliter. In descriptive analyses, vitamin D levels were categorized as sufficient ($\geqslant$30 ng/ml), insufficient ($\geqslant$20 and <30 ng/ml) and deficient (<20 ng/ml) on the basis of previous recommendations [24].

2.4. Statistical analysis

All genotypes were tested for Hardy–Weinberg equilibrium. Association analysis was performed using standard Chi-squared test (Epistat statistical package, Epi Info Version 6) to detect