ORIGINAL ARTICLE

(CCTTT)n Polymorphism of *NOS2A* in Nasal Polyposis and Asthma: A Case-Control Study

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Abstract

Background: Nitric Oxide (NO) has been proposed as an important signaling molecule. NO produced by the inducible NO synthase enzyme NOS2A is generated at high levels in certain types of inflammation. A pentanucleotide polypyrimidine microsatellite CCTTT has been identified in the promoter region of the *NOS2A* gene.
Objective: The aim of this study was to analyze the (CCTTT)n polymorphism in patients with asthma and nasal polyposis.
Material and methods: The study included 292 white individuals (194 patients and 98 controls). Asthma was diagnosed according to American Thoracic Society criteria and classified in accordance with the guidelines of the Global Initiative for Asthma. Skin prick tests were performed in all individuals. The polymorphism was analyzed by an electrophoretic method and by direct sequencing.
Results: A significant association was detected for a 15-repeat cutoff in nasal polyposis (Fisher *P* value = .0001, Monte Carlo *P* value [after 10^4 simulations] = .002). Multivariate analysis adjusted for age and sex confirmed this association with an increased risk of nasal polyposis (odds ratio, 14.39; 95% confidence interval, 3.02 - 68.60; *P* = .001).
Conclusion: The number of CCTTT repeats in the promoter region of *NOS2A* could be associated with the inflammatory process of nasal polyposis in our population. Modifications of *NOS2A* transcription levels could be involved in this association.

Key words: Asthma. Gene. Nasal polyposis. *NOS2A*. Polymorphism. Promoter.

Resumen

Antecedentes: El Óxido nítrico (NO) es una de las moléculas más importantes implicadas en la transmisión de señales. En ciertos tipos de inflamación, los niveles de NO llegan a alcanzar valores muy elevados debido a un aumento en los niveles de transcripción de la sintasa inducible de NO (NOS2A), una de las enzimas responsables de la síntesis de esta molécula. El microsatélite de repetición (CCTTT)n localizado en la región promotora del gen *NOS2A* es un elemento regulador de la transcripción de esta enzima.
Objetivo: En este estudio nos propusimos analizar la distribución del polimorfismo (CCTTT)n en una población de pacientes con asma y poliposis nasosinusal.
Métodos: Se seleccionaron 292 individuos (194 pacientes y 98 controles). El diagnóstico de asma se realizó siguiendo los criterios de la American Thoracic Society y la gravedad se clasificó según las recomendaciones del Global Initiative for Asthma. Se realizaron pruebas cutáneas de hipersensibilidad inmediata a todos los individuos.
Resultados: Detectamos una asociación entre la presencia de quince o más repeticiones del microsatélite de repetición (CCTTT)n y la poliposis nasal (*P* de Fisher = 0,0001, *P* de Monte Carlo tras 10^4 simulaciones = 0,002]. El análisis multivariante ajustado por edad y sexo confirmó esta asociación, con un incremento en el riesgo de padecer pólipos nasales (cociente de posibilidades, 14.39; intervalo de confianza 95%, 3.02 - 68.60; *P* = 0,001).
Conclusiones: El número de repeticiones del pentanucleótido CCTTT en la región promotora del gen *NOS2A* parece estar asociado al proceso inflamatorio que subyace en las poliposis nasales. Posibles modificaciones en los niveles de transcripción del gen *NOS2A* podrían estar implicadas en esta asociación.

Palabras clave: Asma. Gen. Poliposis nasal. *NOS2A*. Polimorfismo. Promotor.

Introduction

Nasal polyps are semitranslucent edematous masses with a broad or slim base usually arising from the mucosal linings of the paranasal sinuses. Most theories consider polyps to be the manifestation of a chronic inflammation. Nitric oxide (NO) has been proposed as an important intracellular and intercellular signaling molecule. NO is produced by NO synthase (NOS). The inducible form of this enzyme, encoded by the gene *NOS2A*, generates high levels of NO in certain types of inflammation and it has been implicated as a proinflammatory agent [1]. The paranasal sinuses constitute the major source of nasal NO, mostly through *NOS2A* [2].

Both exhaled NO and epithelial expression of *NOS2A* are increased in subjects with asthma [3]. The enzyme is produced as a cytotoxic response mechanism by a wide variety of cells. *NOS2A* has been mapped to chromosome 17cen-q11.2 [4]. Genome–wide searches have suggested the importance of chromosomal region 17q11.2-q12 in asthma and atopy susceptibility [5,6].

A pentanucleotide polypyrimidine microsatellite CCTTT at position -2.6 kb has been identified in the promoter region of *NOS2A* [7]. It has been reported that differences in *NOS2A* expression could depend on the number of repeats for CCTTT [8]. This polymorphism has been studied in relation to a variety of diseases such as malaria [9], diabetic retinopathy [8], diabetic nephropathy [10], human immunodeficiency virus infection [11], and specifically asthma and atopy [12-14]. It has been revealed that there is a significantly increased prevalence of the 14-repeat allele of the variable number tandem repeat in nonatopic subjects [12]. Considering that most NO from the upper airways in chronic inflammation is synthesized by *NOS2A* [15], the aim of this study was to analyze the (CCTTT)n polymorphism of *NOS2A* in patients with nasal polyposis and/or asthma.

Materials and Methods

Study Subjects

A total of 292 unrelated white individuals were included in the study: 194 patients and 98 healthy controls from the Allergy Department of the University Hospital of Salamanca. The study was approved by the Ethics Committee of the University Hospital of Salamanca and written informed consent was obtained in all cases.

Patients with physician-diagnosed asthma were recruited. The inclusion criteria were as follows: at least 2 symptoms consistent with asthma (cough, wheeze, and dyspnea); bronchial hyperresponsiveness, defined by a positive bronchodilator test or a positive methacholine challenge test; and absence of other pulmonary disorders. Lung function was measured by spirometry according to American Thoracic Society criteria [16].

Individuals were enrolled as controls when meeting all the following criteria: absence of symptoms or history of asthma; no symptoms or history of other pulmonary diseases; no symptoms or history of nasal polyps; no symptoms or history of allergy; negative skin prick tests to a battery of common aeroallergens (wheal <1 mm greater than saline); and absence of first-degree relatives with a history of asthma or atopy. Control individuals were older, in order to permit a longer period for an asthma diagnosis to be made.

Following European Academy of Allergology and Clinical Immunology guidelines [17], skin tests were considered positive if at least 1 wheal reaction of more than 3 mm diameter after subtraction of negative control was observed in skin prick tests with a battery of common aeroallergens, as previously described [18]. Histamine (10 mg/mL) was used as positive control and saline as negative control. Allergic status was based on a positive skin test to at least 1 allergen of the common aeroallergens.

Polyps were diagnosed according to the guidelines of the European Position Paper on Rhinosinusitis and Nasal Polyps [19], according to which the patients should report 2 or more of the following symptoms: nasal blockage/congestion, discharge, anterior/posterior nasal drip, facial pain/pressure, and reduction or loss of smell. Reduction or loss of smell was considered an essential symptom. Patients with cystic fibrosis, gross immunodeficiency, congenital mucociliary complaints, noninvasive fungal balls, invasive fungal disease, or systemic vasculitic or granulomatous diseases were excluded from the study.

(CCTTT)n Polymorphism Analysis

Genomic DNA was isolated from peripheral whole blood using a DNA extraction kit (Genedan, Barcelona, Spain) [20]. Polymerase chain reaction (PCR) amplification of the corresponding fragments from the *NOS2A* promoter region was performed in a MWG-BIOTHECH thermal cycler (Biothech, Ebersberg, Germany). To amplify the genomic region surrounding the (CCTTT)n polymorphism, the following primers were used: 5'-ACCCCTGGAAGCCTACAACTGCAT-3' and 5'-GCCATCGCACCCTAGCCTGTCTCA-3' [11]. The reaction was performed in a final volume of 25 μL including 12.5 μL of a commercial PCR master mix (Promega, Madison, Wisconsin, USA), 1.25 pmol of each primer, and 20 ng of genomic DNA. Cycling conditions included 1 cycle of 94ºC for 10 minutes; 40 cycles at 94ºC for 1 minute, 60ºC for 1 minute, and 72ºC for 1 minute; and a final extension at 72ºC for 10 minutes. To avoid contamination, negative controls were included in each PCR reaction. Samples were initially analyzed by direct sequencing in a 3100 ABI PRISM Genetic Analyzer automated DNA sequencer (Applied Biosystems, Foster City, California, USA). The resulting chromatograms were aligned and viewed using Chromas 2.3 (Technelysium Pty Ltd, Ewantin, Australia). The GenBank accession number for the reference genomic sequence of *NOS2A* is AF440785. To confirm these results, an electrophoretic method was also used. PCR was performed using a forward-labeled 6-FAM primer. The PCR products were separated through a POP-4 gel using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems); TAMURA 500 was the internal size standard. Allele sizes were calculated through use of the Peak Scanner program (Applied Byosistems).

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guidelines were followed in the laboratory procedures [21,22]. Control subjects and patients were genotyped in a single batch. Two independent scientists performed the analysis blindly with respect to case and control status.

Statistical Analysis

The overall distribution of alleles was analyzed in control and patient groups. In addition, different cutoffs (11, 12, 13, 14, and 15 repeats) were studied. For each cutoff, alleles with fewer repeats were defined as short (S) and alleles with an equal or larger number of repeats were defined as long (L). The subjects were classified into 3 groups: S homozygous (S/S), heterozygous (S/L), and L homozygous (L/L).

Hardy-Weinberg equilibrium was estimated by χ^2 test. The dichotomous variables were analyzed using χ^2 test and the Fisher exact test on contingency tables for distribution of categorical variables. Monte Carlo simulation and Bonferroni correction for multiple comparisons was provided when required. Analysis of variance was used to compare continuous variables across the levels of each genotype. Immunoglobulin (Ig) E levels were transformed to log10 values in order to provide a normal distribution for statistical analysis. Linear regression was used to model the effects of multiple covariates on the continuous and dichotomous outcomes. In multivariate analysis, age and sex were included as potential covariates. A *P* value of .05 or less was considered statistically significant. All statistical analyses were performed using SPSS version 13.0 (SPSS Inc, Chicago, Illinois, USA) and SHEsis software [23]. Statistical power [24] and false-positive report probability (FPRP) [25] were also calculated.

Results

The characteristics of the study population are shown in Table 1.

Allele sizes varied form 7 to 17 repeats in our population. The overall distribution of the polymorphism is shown in the Figure. The most frequent allele contained 12 repeats. The (CCTTT)n genotype frequencies did not deviate from the expected value by Hardy-Weinberg equilibrium.

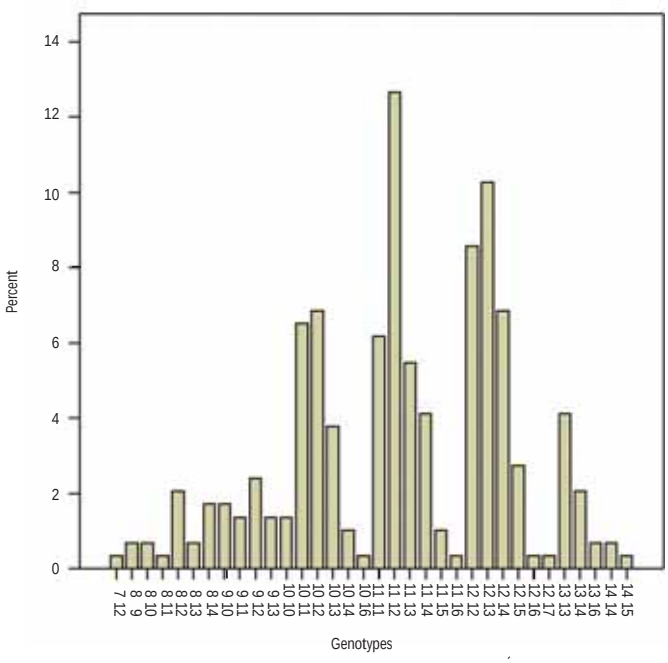

Figure. Distribution of genotype frequencies of (CCTTT)n polymorphism of *NOS2A* gene in our population.

For the overall distribution of alleles, a slight statistical difference was observed between the control group and the group of patients with nasal polyposis (Fisher *P* value = .03, Monte Carlo *P* value [after 10^4 simulations] = .02) (Table 2).

Table 1. Demographic Characteristics of Control Subjects, Patients With Asthma, and Patients With Polyposis[a]

	Controls (n = 98)	Asthma (n = 150)[b]	Polyposis (n = 46)
Age, y	46 (18)	32.3 (16.6)	50.17 (13.74)
Sex, No., Female/Male	67/31	82/68	20/26
log IgE, kU_A/L	1.50 (0.56)	2.24 (0.62)	1.88 (0.53)

Abbreviation: IgE, immunoglobulin E.
[a] Data are shown as mean (SD) unless otherwise indicated.
[b] Atopic asthma, n = 103; nonatopic asthma, n = 47

Table 2. Allele Frequencies for the Different Repeats in Patients With Polyposis, Atopic Asthma, and Nonatopic Asthma, and in Healthy Control Subjects

No. of Repeats	Controls (196 alleles)	Polyposis[a] (92 alleles)	Atopic (204 alleles)	Nonatopic (94 alleles)
7	0.005	–	–	–
8	0.036	0.043	0.025	–
9	0.041	0.011	0.034	0.032
10	0.122	0.120	0.088	0.128
11	0.245	0.141	0.245	0.223
12	0.296	0.315	0.343	0.266
13	0.158	0.185	0.123	0.213
14	0.082	0.087	0.118	0.064
15	0.010	0.065	0.015	0.043
16	–	0.033	0.010	0.032
17	0.005	–	–	–

[a] Fisher *P* value =.03 and Monte Carlo *P* value (after 10^4 simulations) =.02 compared to control

Table 3. Allele Frequencies of (CCTTT)n Polymorphism for Different Repeat Cutoffs

Repeat Cutoffs	Control (196 alleles)		Polyposis (92 alleles)		Monte Carlo *P* Value (controls/ polyposis)
	S	L	S	L	
11	0.20	0.80	0.17	0.83	.641
12	0.45	0.55	0.31	0.69	.040
13	0.75	0.25	0.63	0.37	.053
14	0.91	0.09	0.81	0.19	.032
15	0.99	0.01	0.90	0.10	.002

Abbreviations: S, short allele (No. of repeats < cutoff); L, long allele (No. of repeats > cutoff).

A specific analysis of the different cutoffs for number of repeats is shown in Table 3. A significant association was detected in allele and genotype frequencies for a 15-repeat cutoff in nasal polyposis (Fisher *P* value = .0001, Monte Carlo *P* value [after 10^4 simulations] = .002 for genotype frequencies). Multivariate analysis adjusted for age and sex confirmed this association with an increased risk of nasal polyposis (odds ratio, 14.39; 95% confidence interval, 3.02 - 68.60; *P* = .001). Considering our own population as a reference, the statistical power would be more than 80% with an α error less than 0.05. The FPRP would be less than 5% for a pre-test probability of 0.1.

An association with nonatopic asthma was observed for a 15-repeat cutoff (Fisher *P* value = .008, Monte Carlo *P* value [after 10^4 simulations] = .012). No association with atopic asthma was detected (Table 2).

Discussion

NO is an important intracellular and intercellular signaling molecule involved in the regulation of diverse physiological and pathophysiological processes in the cardiovascular, nervous, and immune systems [26]. It is difficult to determine in exactly which processes NO is involved because its toxic effects run in parallel with its protective activity. The paranasal sinuses constitute the major source of nasal NO, mostly synthesized by *NOS2A* [2]. In addition, it has been described that differences in *NOS2A* expression could depend on the number of CCTTT repeats [2,8], although conflicting results have been reported [27].

In our population, the most frequent allele contained 12 repeats, consistent with previously reported data in other European populations [2,28]. We detected a relationship between the (CCTTT)n polymorphism in the promoter region of *NOS2A* gene and nasal polyposis in our population. An interesting study performed by Warpeha et al [8] demonstrated the functional role of the CCTTT repeat. They found that 9, 12, and 14 repeats yielded a progressive increase in gene expression. As reported by other authors [14], we assume that as NOS2A is the predominant NO-producing enzyme in inflammation, this enzyme could account for all NO produced. We developed a progressive analysis taking into account different repeat cutoffs (11, 12, 13, 14, and 15 repeats) and found a continuous increase in the number of repeats associated with nasal polyposis, with a specific cutoff at 15 repeats.

In addition, we studied the relationship between this polymorphism and asthma and atopy. We did not find any association with atopic asthma. In recent studies, no association of this polymorphism with asthma has been detected [12-14]. Konno et al [12] reported an increased prevalence of 14 repeats in nonatopic patients. The authors established the comparison between 14 repeats against all other numbers of repeats. In fact, they included cases with more than 14 repeats and cases with less than 14 repeats in the same group. Considering the different contribution that short and long alleles seem to exert, we believe that this approach could mask the real cumulative effect of repeat number. We detected a certain association with nonatopic asthma; however, more than 40% of patients with nonatopic asthma suffered from polyposis, and this could have partially contributed to the association in this group.

The pathogenic mechanism underlying the association with nasal polyposis is not clear. Two characteristics of nasal polyposis in adults are their strong association with tissue eosinophilia, which can be observed in about 70% to 90% of patients [15], and the increased expression of *NOS2A* [29]. A close relationship between nitric oxide and eosinophils has been described because it is known that NO disrupt the apoptotic Fas signal in eosinophils [30] and might act as a chemoattractant factor for them [31], extending the inflammatory status. It is known that human eosinophils can express mRNA for *NOS2A*, and eosinophils could be one of the sources of the increased NO production [32].

Eastmont and coworkers [33] showed that interferon γ is able to regulate, via NO, the secretory activity of mast cells, by suppressing IgE secretion [29]. These findings might help to explain the absence of a relationship with the atopic asthma population even if a definitive explanation cannot be provided.

In summary, we detected an association between the (CCTTT)n polymorphism of the *NOS2A* gene and nasal polyposis in our population. To our knowledge this is the first genetic association study of *NOS2A* in patients with polyposis. Confirmation of our findings by studies in other populations could provide a better understanding of the genetic significance of this polymorphism in the development of nasal polyposis.

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