Allergology International 65 (2016) 300–305

Contents lists available at ScienceDirect

Allergology International

journal homepage: http://www.elsevier.com/locate/alit

Original article

Exhaled nitric oxide and inducible nitric oxide synthase gene polymorphism in Japanese asthmatics

Suguru Sato*, Xintao Wang, Junpei Saito, Atsuro Fukuhara, Manabu Uematsu, Yasuhito Suzuki, Yuki Sato, Kenichi Misa, Takefumi Nikaido, Naoko Fukuhara, Yoshinori Tanino, Mitsuru Munakata

Department of Pulmonary Medicine, School of Medicine, Fukushima Medical University, Fukushima, Japan

ARTICLE INFO

Article history:
Received 5 November 2015
Received in revised form
12 February 2016
Accepted 15 February 2016
Available online 23 March 2016

Keywords:
Adult asthma
Asthma
Fractional exhaled nitric oxide
Genetic polymorphism
Single nucleotide polymorphism

List of abbreviations:
FeNO, fractional exhaled nitric oxide; iNOS, inducible nitric oxide synthase; NOS2A, inducible nitric oxide synthase; SNPs, single nucleotide polymorphisms; ICS, inhaled corticosteroids; ATS, American Thoracic Society; PCR, polymerase chain reaction; SEM, standard error of the mean; FVC, forced expiratory volume; FEV_1, forced expiratory volume in one second; FEV_1/FVC, forced expiratory volume in one-second percent

ABSTRACT

Background: Inducible nitric oxide synthase (iNOS) induced by inflammatory cytokines and iNOS activity in bronchial epithelial cells is a major determinant of fractional exhaled nitric oxide (FeNO) levels. The aim of this study was to investigate the association of iNOS promoter gene polymorphisms and FeNO levels in Japanese asthmatics before the introduction of asthma treatment.
Methods: Asthmatics were recruited from Fukushima Medical University Hospital. Genotyping of the pentanucleotide repeat (CCTTT)n and seven previously detected single nucleotide polymorphisms (SNPs) in the iNOS promoter lesion was performed. The relationships between the genotypes and FeNO levels before the introduction of asthma treatment were compared.
Results: In 91 asthmatics, the number of microsatellite repeats ranged from 9 to 20 and showed a bimodal distribution. According to this distribution, asthmatics were divided into two groups: genotypes with at least one long allele with more than 14 repeats (*L/s or L/L*) and genotypes with both short alleles with 14 or fewer repeats (*s/s*). No significant differences were observed in each parameter between the two groups. The mean FeNO level before treatment was significantly higher in the *L/s or L/L* subjects than in the *s/s* subjects. After treatment, the lowest FeNO level did not differ between the two groups. Three SNPs detected in the Japanese subjects were not associated with FeNO levels.
Conclusions: The number of CCTTT repeats in the iNOS promoter region was associated with FeNO levels in asthmatics before treatment, suggesting the importance of iNOS genotype in the clinical application of FeNO for asthmatics.

Introduction

Fractional exhaled nitric oxide (FeNO) is a useful diagnostic tool for bronchial asthma.[1–3] NO is produced by a wide variety of cells and is generated through conversion of L-arginine to L-citrulline by nitric oxide synthase.[4] In addition, inducible nitric oxide synthase (iNOS) is activated by inflammatory cytokines, and iNOS activity in bronchial epithelial cells is a major determinant of FeNO level.[5]

The inducible-NOS gene (*NOS2A*) is present on chromosome 17q11.2–12, which comprises 27 exons, with the transcription start site in exon 2 and stop codon in exon 27.[6] *NOS2A* is predominantly transcriptionally regulated, therefore genetic variations within the 5′ region may influence gene expression. The proximal *NOS2A* promoter contains pentanucleotide microsatellites and single nucleotide polymorphisms (SNPs). These polymorphisms play important roles in several diseases. Pentanucleotide (CCTTT) polymorphisms in the promoter regions of *NOS2A* and other SNPs have been investigated in various diseases including asthma, rheumatoid arthritis, malaria, and inflammatory bowel diseases.[7] Konno *et al.* reported that a 14-repeat allele is inversely associated with atopy.[8] Pascual *et al.* reported in their case control study that the number of repeats could be associated with the inflammatory process of nasal

* Corresponding author. Department of Pulmonary Medicine, School of Medicine, Fukushima Medical University, Hikarigaoka-1, Fukushima 960-1295, Japan.
E-mail address: suguru@fmu.ac.jp (S. Sato).
Peer review under responsibility of Japanese Society of Allergology.

http://dx.doi.org/10.1016/j.alit.2016.02.007

polyposis.[9] SNPs at position −954 G/C, −1173 C/T, and −1659 A/T in the *NOS2A* promoter region have been shown to increase NO synthesis.[10] Batra *et al.* reported the association of *NOS2A* polymorphism with severity of asthma and eosinophils. They also found an association between serum nitric oxide levels and *NOS2A* promoter repeats.[11] The association between levels of FeNO and polymorphisms in *NOS2A* has previously been reported. A recent population-based study found SNPs in *NOS2A* to be significantly associated with FeNO, and the association was particularly strong in asthmatic children.[12] In a Swedish study of an adult general population, two SNPs in *NOS2A* and one in *NOS3* revealed an independent association with levels of FeNO. However, this association varied in asthmatics, and no significant association was found between SNPs in *NOS2A* and levels of FeNO.[13]

It should be noted that the majority of these studies were done mainly for asthmatic patients who were already being treated with inhaled corticosteroids, and few reports exist examining the influence of polymorphisms in the *NOS2A* promoter region and FeNO levels at diagnosis before the introduction of asthma treatment. For this reason, in the present study we analyzed the proximal *NOS2A* promoter pentanucleotide microsatellite and other SNPs, and studied the association between the polymorphisms and the levels of FeNO in asthmatics before treatment. Moreover, change in FeNO level after asthmatic treatment was analyzed.

Methods

Study subjects and study design

Present study was a retrospective observational study. Asthmatic subjects were recruited from the outpatient clinic at the Department of Pulmonary Medicine in Fukushima Medical University Hospital between September 2009 and December 2012. They had no abnormalities on chest X-ray and did not receive anti-asthma therapies, including inhaled and systemic corticosteroids, leukotriene receptor antagonists, theophylline, and omalizumab. All patients had been diagnosed according to the American Thoracic Society (ATS) criteria.[14] Asthma was defined on a basis of recurrent episodes of at least one symptom (cough, wheeze, or dyspnea) associated with a demonstrated reversible airflow limitation (12% and 200 mL variability in forced expiratory volume in 1 s [FEV_1] either spontaneously or with an inhaled short-acting β_2-agonist) and/or increased airway responsiveness. Asthmatics who had upper or lower airway infection, malignancy, or collagen vascular disease at initial assessment were excluded from the study. Asthma severity was assessed according to the Global Initiative of Asthma 2012 guidelines, and classified into four groups: mild intermittent, mild persistent, moderate persistent and severe persistent. A concomitance of chronic sinusitis was based on the patient's report or the presence of two or more of the following symptoms: nasal blockage/congestion, discharge, anterior/posterior nasal drip, facial pain/pressure, and reduction or loss of smell. All subjects provided written informed consent for both the analysis of their clinical data and the genotyping of their DNA extracted from peripheral blood. If subjects were under the age of 18, they and their guardians had to provide written informed consent prior to enrollment. This study was approved by the ethics committee of Fukushima Medical University on June 20, 2000 [No.65].

For initial assessment, FeNO measurement, blood tests, and pulmonary function tests were performed, and asthma severity was classified. All current smokers in this study stopped smoking at least two weeks before the initial assessment. Following the initial assessment, all recruited subjects were administered asthmatic treatment including inhaled corticosteroids (ICS) in accordance with treatment guidelines. During the treatment, we also performed FeNO measurement for monitoring asthma control. We defined: 'FeNO level before treatment' as the patient's FeNO level at diagnosis; 'FeNO level after treatment' as the minimum FeNO level during the treatment periods; and 'ΔFeNO' as the difference between 'FeNO level before treatment' and 'FeNO level after treatment.' According to the ATS clinical practical guidelines, 'high FeNO level' in adults is defined as a level higher than 50 ppb.[15]

FeNO measurement

FeNO was measured in accordance with ATS and European Respiratory Society recommendations[15] using a chemiluminescence analyzer NA623N® (Kimoto, Osaka, Japan), and is expressed as parts per billion. NA623N® is a stationary FeNO analyzer, and the measurement values of FeNO strongly correlated with the values measured by a widely-used portable FeNO analyzer, NIOX-MINO® (Aerocrine, AB, Solna, Sweden).[16] Measurement was performed as described previously.[17] In brief, FeNO was measured with patients in a sitting position and without the use of a nose clip. From total lung capacity without holding their breath, the patient exhaled at a constant flow of 50 mL/s. FeNO was measured three times, with differences in measured values within 10%. The mean value of the three measurements was used for statistical analysis.

Blood tests and pulmonary function test

Blood tests included peripheral blood eosinophil count, serum non-specific IgE, and antigen-specific IgE. A radioallergosorbent test for antigen-specific IgE was performed for weeds, mites, house dust, cats, dogs, cedar, cypress, orchard grass, moths, *Aspergillus*, *Candida*, and mixed molds. Non-specific IgE was measured by fluorescence enzyme immunoassay (UniCAP; Pharmacia & Upjohn, Uppsala, Sweden). Atopy was defined as either a non-specific IgE concentration greater than 250 IU/mL or any positive antigen-specific IgE (higher than 0.70 UA/mL). Pulmonary function testing was performed using rolling seal spirometers (Chestac-11 Cyber S-type; Chest MI, Inc., Tokyo, Japan) to measure forced expiratory volume (FVC) and FEV_1. Tests were performed by experienced respiratory technicians according to ATS guidelines.[18] The FVC and FEV_1 are expressed as percent of predicted values.

Genotyping and analyses

Genotyping of the pentanucleotide repeats, (CCTTT)n in the *NOS2A* promoter region, was performed by polymerase chain reaction with appropriate primers (sense primer, 5′-ACC CCT GGA AGC CTA CAA CTG CAT-3′; anti-sense primer, 5′-GCC ACT GCA CCC TAG CCT GTC TCA-3′), and by a fluorescently labeled primer method and capillary gel electrophoresis with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Allele distribution was analyzed, and asthmatics were divided into two groups: those with alleles with higher numbers of repeats, and all other genotypes. Xu *et al.* reported on the allelic frequency of *NOS2A* promoter pentanucleotide microsatellite in 210 Japanese individuals.[19] In the Japanese population, alleles showed a bimodal distribution pattern. Furthermore, previous reports on the relationship between the *NOS2A* promoter pentanucleotide repeat and fatal malaria in the Gambian and Tanzanian populations defined allele length based on a trough occurring repeat number of a bimodal distribution.[20,21] We defined that the cut-off number of repeats for distinguishing lower and higher repeat was a trough occurring repeat number of a bimodal distribution referred to these reports. Characteristics and FeNO levels before treatment were compared between the two groups.