

Association between (CCTTT)*n* repeat polymorphism in *NOS2* promoter and asthma exacerbations



To the Editor:

Asthma is a heterogeneous disease characterized by airway inflammation. In allergic asthma, T_H2 cells play a major role in eosinophilic airway inflammation, through T_H2 cytokines including IL-4, IL-5, and IL-13. T_H2 cytokines induce the expression of inducible nitric oxide synthase (NOS2) in bronchial epithelial cells. Thus, the fractional exhaled nitric oxide (FENO) level is a useful surrogate biomarker for eosinophilic airway inflammation.

Previous studies suggested that the gene expression level of *NOS2* strongly correlates with FENO level,¹ and polymorphisms in the *NOS2* gene affect mRNA expression levels. Pentanucleotide (CCTTT) repeat polymorphism exists in the promoter region of

NOS2 and has demonstrated associations with the prevalence of atopy and nasal polyposis.^{2,3} An association of CCTTT repeat polymorphism with FENO level was indicated in steroid-naïve patients with asthma⁴; however, its association with the treatment outcomes of patients with asthma remains unclear. This study aimed to investigate the association of CCTTT repeat polymorphism on *NOS2* promoter with exacerbations in patients with asthma.

Subjects included 136 patients with moderate to severe asthma from the previous study performed in Shizuoka General Hospital between February and December 2015.⁵ All patients with asthma fulfilled the definition given by the Global Initiative for Asthma (GINA) and were categorized as having moderate (GINA step 3) or severe (GINA step 4-5) asthma. Study subjects underwent evaluation to determine *NOS2* mRNA expression levels in PBMCs, FENO levels, plasma nitric oxide metabolite nitrate and nitrite (NO_x) concentrations, white blood cell counts, and lung

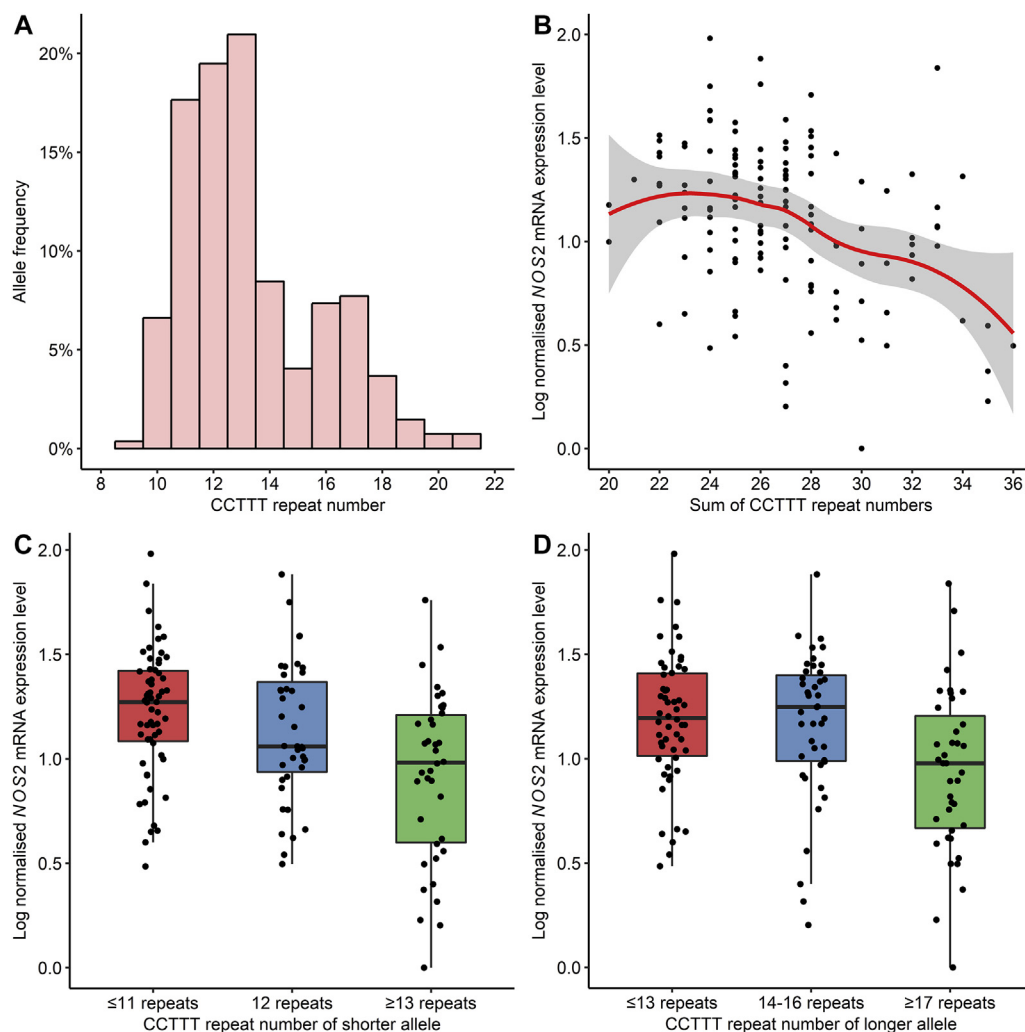


FIG 1. The relationship between the CCTTT repeat polymorphism and *NOS2* mRNA expression levels. **A**, The allele frequency of CCTTT repeat polymorphisms in *NOS2*. **B**, The smoothing line for changes in *NOS2* mRNA expression levels by the sum of CCTTT repeat number. **C**, *NOS2* mRNA expression levels across the shorter allele. **D**, *NOS2* mRNA expression levels across the longer allele.

TABLE I. Comparison between subjects' characteristics with and without CCTTT ≤ 11 repeats allele

Characteristic	All subjects (n = 136)	S/S + S/L (n = 59)	L/L (n = 77)	P value
Age (y)	66 (50-74)	64 (45-73)	67 (54-75)	.254
BMI (kg/m ²)	23.1 (20.7-25.4)	23.9 (20.7-26.0)	22.8 (20.7-25.2)	.213
Sex: male	63 (46)	26 (44)	37 (48)	.644
Childhood onset	25 (18)	14 (24)	11 (14)	.184
GINA treatment step				
Step 5	13 (10)	8 (14)	5 (6)	.375
Step 4	76 (56)	32 (54)	44 (57)	
Step 3	47 (35)	19 (32)	28 (36)	
Atopy	101 (74)	47 (80)	54 (70)	.208
Allergic rhinitis	81 (60)	36 (61)	45 (58)	.762
Smoking status				
Current smoker	4 (3)	2 (3)	2 (3)	.900
Ex-smoker	58 (43)	24 (41)	34 (44)	
Never smoker	74 (54)	33 (56)	41 (53)	
Pack-years	0 (0-16)	0 (0-17)	0 (0-15)	.752
FEV ₁ % predicted	83.4 (68.1-95.4)	86.1 (71.8-95.6)	81.9 (67.1-94.9)	.283
FEV ₁ /FVC	69.5 (62.5-76.1)	71.7 (65.7-78.6)	67.7 (60.8-74.8)	.038
FENO (ppb)	30.0 (16.9-48.2)	29.3 (15.1-55.6)	30.8 (19.5-47.7)	.683
Total IgE (IU/mL)	239 (88-644)	274 (91-986)	233 (84-558)	.351
NOS2 mRNA level	14.5 (8.2-22.2)	18.7 (12.1-26.4)	11.2 (6.6-20.1)	.001
Plasma NO _x (ng/mL)	38.6 (28.6-60.7)	36.6 (27.4-61.9)	42.5 (30.7-55.9)	.445
Peripheral blood cells (cells/ μ L)				
Neutrophil	3602 (2975-4636)	3548 (3065-4995)	3743 (2930-4516)	.928
Lymphocyte	1683 (1371-2100)	1684 (1362-2087)	1670 (1373-2101)	.696
Monocyte	367 (300-461)	364 (293-467)	368 (306-461)	.836
Eosinophil	337 (206-521)	316 (213-553)	351 (202-509)	.802
Basophil	43 (31-63)	41 (31-71)	49 (33-61)	.816
ICS dose (μ g/d)	320 (200-640)	400 (225-640)	320 (200-640)	.472
Oral corticosteroid	10 (7)	6 (10)	4 (5)	.330
Exacerbation	39 (29)	23 (39)	16 (21)	.020

BMI, Body mass index; FVC, forced vital capacity.

Data are shown as median (interquartile range) or frequency (percentage). ICS daily dose expressed as equivalent dose to fluticasone propionate. Childhood onset, defined as onset before age 16 years. The alleles shorter than or equal to 11 repeats are indicated by short alleles (S) and those longer than 11 repeats are indicated by long alleles (L).

function in a cross-sectional study. Exacerbations, defined as a worsening of symptoms requiring treatment with systemic corticosteroids, were recorded 1 year before and after the cross-sectional study. This study was approved by the Ethics Committee of the Shizuoka General Hospital (SGH 15-01-55). Relative expression levels of NOS2 mRNA were quantified using real-time PCR with SYBR Green detection and normalized to β -actin expression. NOS2 CCTTT repeat polymorphism genotyping was performed using PCR followed by capillary electrophoresis. Logistic regression analysis was used to calculate the odds ratios (ORs) for exacerbation and 95% Wald CI with and without adjustment for age, body mass index, sex, and FEV₁% predicted. The detailed Methods are available in this article's Online Repository at www.jacionline.org.

The NOS2 CCTTT repeat number ranged from 9 to 22, with 13 being the most frequent allele (Fig 1, A). The relationship between the sum of CCTTT repeat number and NOS2 mRNA expression levels is presented in Fig 1, B. The smoothed line indicated that the expression levels of NOS2 mRNA gradually decreased as the repeat number increased ($P < .001$ by F test). This trend was maintained when subjects were subdivided according to the CCTTT repeat number of the shorter ($P < .001$) or longer allele ($P = .003$) (see Fig E1 in this article's Online Repository at www.jacionline.org). These gradual changes were also found in study subjects who were divided into 3 groups by tertiles of repeat number for the shorter allele

($P < .0001$ by the Jonckheere-Terpstra test, Fig 1, C) and the longer allele ($P = .002$; Fig 1, D). NOS2 mRNA expression levels were the highest in patients with the shorter allele having 11 or fewer repeats. When we classified the alleles shorter than or equal to 11 repeats as short alleles (S) and those longer than 11 repeats as long alleles (L), subjects having a short allele (S/S and S/L) were associated with a higher risk of exacerbation (Table I) (unadjusted OR, 2.4; 95% CI, 1.1-5.3; and adjusted OR, 2.8; 95% CI, 1.2-6.6). An independent association of CCTTT repeat polymorphism almost remained even when subjects were stratified by age at onset of asthma (see Table E1 in this article's Online Repository at www.jacionline.org); however, there was no independent relationship between the expression levels of NOS2 mRNA and exacerbation (unadjusted OR, 1.2; 95% CI, 0.6-2.7; and adjusted OR, 1.2; 95% CI, 0.5-2.9).

This study is the first to characterize CCTTT repeat polymorphism with the clinical outcomes of asthma; however, we have not elucidated the independent contribution of NOS2 mRNA expression levels to exacerbation. This discrepancy may be due to our cross-sectional study design, which evaluated the expression levels of NOS2 mRNA at the one-time point in PBMCs; furthermore, it is important to consider the epigenetic regulation of NOS2 mRNA expression.⁶ Nevertheless, it should be noted that we identified a novel nonlinear relationship between the repeat number of CCTTT and the NOS2 mRNA expression

levels in PBMCs of patients with asthma. This result is supported by previous *in vivo* studies demonstrating that *NOS2* promoter activities were the highest in cells transfected with 12 or 14 CCTTT repeats, as indicated by bell-shaped activity profiles.^{7,8} Although it is quite conceivable that CCTTT repeat polymorphism influences asthma exacerbation through changing the level of gene expression, the pathophysiological role of this polymorphism in the inflammatory processes should be uncovered in further studies, which evaluate the *NOS2* mRNA expression level either in airway cells or in a longitudinal investigation.

In this study, there were no significant differences between the 2 genotype groups in terms of FENO levels or plasma NO_x concentrations. A recent investigation demonstrated that CCTTT repeat polymorphism in *NOS2* contributes to FENO levels in inhaled corticosteroid (ICS)-naïve patients with asthma⁴; however, another study reported no significant association between polymorphism and FENO levels in pediatric patients with asthma, approximately half of whom received ICS treatment.⁹ Although these studies differ in population and the means of division into genotype groups, our results may be attributed to the influence of ICS treatment on nitric oxide synthesis in bronchial cells, regardless of polymorphisms of *NOS2*.

In conclusion, the present study demonstrates the association between pentanucleotide repeat polymorphism in the *NOS2* promoter region and changing gene expression levels. Consequently, it is suggested that having alleles shorter than or equal to 11 repeats contributes to the worsening of clinical outcomes following asthma treatment in patients with moderate to severe asthma.

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***Staphylococcus aureus* enhances the tight junction barrier integrity in healthy nasal tissue, but not in nasal polyps**



To the Editor:

Chronic rhinosinusitis (CRS) is characterized by mucosal inflammation of nasal and sinus cavities. Clinically relevant CRS phenotypes are defined by the presence (CRSwNP) or absence (CRSSNP) of nasal polyps (NPs). CRSwNP is mostly defined by a T_H2-type microenvironment and eosinophilia, whereas CRSSNP rather involves T_H1 responses and neutrophilia.¹ Previous studies have associated *Staphylococcus aureus* as one of the factors driving inflammation in NPs, but the relationship between the mucosal barrier and *S aureus* remains to be investigated.²

Tight junctions (TJs) represent transmembrane and adaptor proteins sealing the most apical part of intercellular junctions between adjacent epithelial cells. They regulate epithelial impermeability against pathogens and foreign particles, such as allergens. Members of the TJ proteins include the occludin, tricellulin, and the claudin families that form homodimers and heterodimers between neighboring cells, forming the intercellular barrier. Junctional adhesion molecules (JAMs) and the zonula occludens (ZO) proteins form the connection of transmembrane TJ molecules to the actin cytoskeleton.³

Defective expression of TJs and barrier leakiness have been reported in allergic disorders, such as asthma,³ atopic dermatitis,⁴ and CRS.⁵ TJs can be regulated by an inflammatory microenvironment in tissues; in CRS, by cytokines such as IFN-γ and IL-4.⁵ Recently, TJs have also been shown to be regulated by TLR-9 ligands, such as CpG-DNA, leading to an increase in TJ expression and epithelial barrier integrity.⁶ TJ genes respond to tissue factors and foreign factors to maintain epithelial integrity; however, the response of epithelial barrier molecules to common airway pathogens remains to be investigated. In this letter, we report that *S aureus* enhances barrier function of healthy nasal epithelial cells by increasing the expression of TJ molecules.

Nasal mucosa tissue explants of human inferior turbinate (IT) from healthy donors and NP tissue from patients with CRSwNP (see Table E1 in this article's Online Repository at www.jacionline.org) were infected with *S aureus* or *Staphylococcus epidermidis* with a dose of 2 × 10⁷ CFU/mL and observed for IL-1β and IL-6 response (see Fig E1, A and B, in this article's Online Repository at www.jacionline.org). *S epidermidis*- and *S aureus*-stimulated samples did not differ in terms of IL-1β and IL-6 expression (Fig E1, C). Samples in

METHODS

Study subjects

Subjects included 136 patients with moderate to severe asthma from the previous study performed in Shizuoka General Hospital between February and December 2015.^{E1} All patients with asthma fulfilled the definition given by GINA and were categorized as having moderate (GINA step 3) or severe (GINA step 4-5) asthma.^{E2} Study subjects underwent evaluation to determine *NOS2* mRNA expression levels in PBMCs, FENO levels, white blood cell counts, and lung function in a cross-sectional study. Exacerbations, defined as a worsening of symptoms requiring treatment with systemic corticosteroids, were recorded 1 year before and after the cross-sectional study. This study was approved by the Ethics Committee of the Shizuoka General Hospital (SGH 15-01-55). Written informed consent was obtained from the patients after the purpose and protocol of the study were explained.

Atopy was defined by positive specific IgE antibodies to at least 1 common inhalant allergen (CAP system; Pharmacia, Uppsala, Sweden). Allergic rhinitis was diagnosed by a history of rhinorrhea, sneezing, nasal congestion, or nasal itching for at least 1 year.

Quantitation of mRNA expression levels

PBMCs were separated from venous EDTA blood with density centrifugation on Ficoll-Paque PLUS (GE Healthcare, Tokyo, Japan) in 15-mL LeucoSep tubes (Greiner Bio-One, Tokyo, Japan), according to the manufacturer's instructions. PBMCs were diluted to 5×10^7 cells/mL and stored at -80°C until use. Total RNA was isolated from PBMCs using the NucleoSpin miRNA kit (Macherey-Nagel, Düren, Germany) and was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, Calif). The real-time quantitative PCR was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems) using the Fast SYBR Green Master Mix (Applied Biosystems). Relative *NOS2* mRNA expression levels were normalized to a β -actin expression level using the $\Delta\Delta\text{Ct}$ method. The cDNA was amplified using forward primer 5'-GCTCCATAGTTTCCAGAAGCAG-3' and reverse primer 5'-GGACCAGC CAAATCCAGTC-3' for 40 cycles of 3 seconds at 95°C and 30 seconds at 60°C . The specificity of the reaction was assessed by an analysis of the melting curve. Each sample was analyzed in duplicate, and any sample with a coefficient of variance of greater than 5% was reanalyzed.

Genotyping for *NOS2* CCTTT repeat polymorphism

Leukocyte genomic DNA was extracted directly from the blood specimens using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Genotyping was performed using PCR followed by capillary electrophoresis.^{E3} Genomic DNA was amplified using forward primer 5'-ACCCCTGGAAGCC TACAACCTGCAT-3', reverse primer 5'-GCCACTGCACCCTAGCCTGTC TCA-3', and HotStar Taq plus DNA polymerase (Qiagen). The forward primer was labeled at the 5' end with the fluorescent dye 6-FAM. The PCR conditions consisted of an initial denaturation at 95°C for 5 minutes, amplification for 35

cycles at 95°C for 30 seconds, 66°C for 30 seconds, and 72°C for 1 minute and a final extension at 72°C for 10 minutes. PCR products were analyzed on ABI PRISM 310 Genetic Analyzer using GeneScan 400HD ROX size standard and the GeneMapper v4.0 software (Applied Biosystems).

Measurement of nitrate and nitrite concentrations

Plasma nitrate and nitrite (NO_x) concentrations were determined using the Griess reaction. Briefly, 20 μL of plasma was mixed with β -nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), and 0.01 U of nitrate reductase and incubated for 60 minutes in a total volume of 100 μL . After that, ZnSO_4 was added to the mixture and centrifuged at 15,000g for 10 minutes. One hundred microliters of Griess reagent was added to 80 μL of supernatant and the mixture was incubated for 10 minutes at room temperature. Absorbance was measured at 550 nm and converted to NO_x content by using a nitrate standard curve.

Statistical analysis

Categorical variables are summarized as frequencies and proportions, and continuous variables are expressed as medians and interquartile ranges. The Kruskal-Wallis test was used to assess differences among 3 groups with continuous variables, and the Mann-Whitney *U* test was applied for pairwise comparisons. The chi-square test or Fisher exact test was used to compare the categorical variables. The association between *NOS2* mRNA expression levels and CCTTT repeat number was evaluated using locally weighted scatterplot smoothing regression. The statistical significance of regression model was evaluated by F test. The Jonckheere-Terpstra test was performed to assess the trend between *NOS2* mRNA expression levels and CCTTT repeat number. On the basis of comparison of *NOS2* mRNA expression levels, we classified the alleles shorter than or equal to 11 repeats as short alleles (S) and those longer than 11 repeats as long alleles (L). Logistic regression analysis was used to calculate the ORs for exacerbation with and without adjustment for age, body mass index, sex, and $\text{FEV}_1\%$ predicted. All analyses were performed with R software (version 3.3.3, R Foundation for Statistical Computing, Vienna, Austria). A 2-tailed *P* value of less than .05 was considered to be statistically significant.

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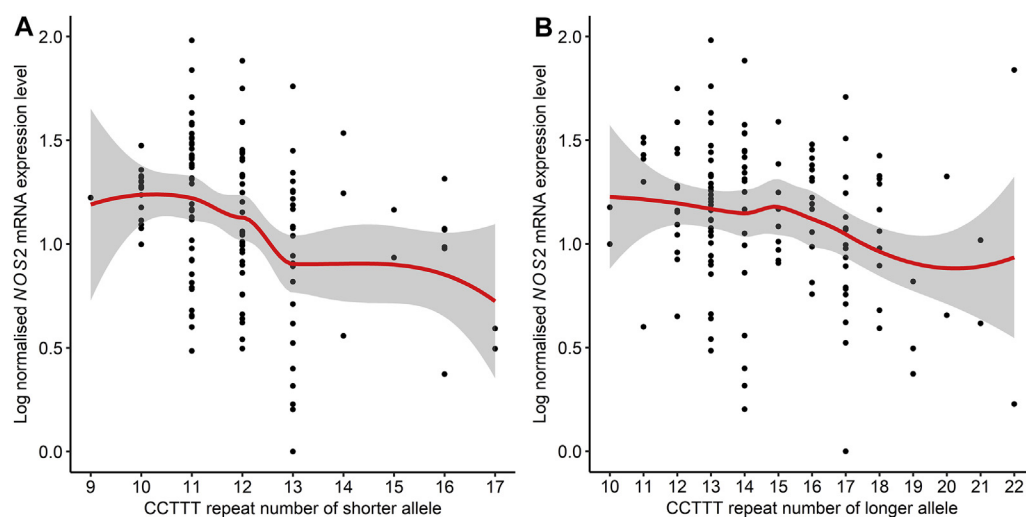


FIG E1. The relationship between the CCTTT repeat polymorphism and *NOS2* mRNA expression levels. **A** and **B**, The smoothing line for changes in *NOS2* mRNA expression levels by CCTTT repeat number of shorter allele (Fig E1, **A**) or CCTTT repeat number of longer allele (Fig E1, **B**).

TABLE E1. OR of *NOS2* CCTTT $\leq$ 11 repeats allele (S/S + S/L) for exacerbation among subjects stratified by age at onset of asthma

Subjects	n	Frequency of exacerbation, n (%)		Unadjusted			Adjusted for age, BMI, sex, and FEV ₁ % of predicted value		
		S/S + S/L	L/L	OR	95% CI	P value	OR	95% CI	P value
All subjects	136	23 (39)	16 (21)	2.4	1.1-5.3	.022*	2.8	1.2-6.6	.016*
Childhood onset	25	6 (43)	1 (9)	7.5	0.7-76	.090†	NA		
Adult onset	111	17 (38)	15 (23)	2.1	0.9-4.8	.088*	2.5	1.0-6.5	.050*

Childhood onset, defined as onset before age 16 years.

BMI, Body mass index; NA, not applicable (because of a small subgroup).

*P values were calculated using the Wald χ^2 test.

†P value was calculated using the Fisher exact test.