CYSTIC FIBROSIS

Nitric oxide synthase 1 as a potential modifier gene of decline in lung function in patients with cystic fibrosis

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Background: The severity of lung disease varies widely in patients with cystic fibrosis (CF) who have the same type of mutations of the cystic fibrosis transmembrane regulator (*CFTR*) gene, suggesting involvement of ''modifier'' genes. The nitric oxide synthase 1 (*NOS1*) gene is a candidate for this role because exhaled nitric oxide (NO) is reduced in patients with CF and NOS1 activity contributes to transepithelial ionic transport, immune defence, and non-specific inflammation of the airways.
Methods: Dinucleotide GT repeat polymorphism was studied in the 5′ untranslated region of the *NOS1* gene, immediately upstream from the transcription initiation site, in 59 patients with CF and 59 healthy controls.
Results: Nineteen alleles of the *NOS1* gene were identified according to the number of GT repeats (from 18 to 36) in the 5 untranslated region. Exhaled NO levels were significantly correlated with the number of GT repeats. Patients with CF who had the *NOS1* genotype associated with high NO production had a slower decline in lung function during the 5 year follow up period. There was no confounding effect of age, chronic bacterial colonisation of the airway, or *CFTR* genotype.
Conclusions: These data suggest a possible link between the *NOS1* gene locus and the rate of decline in lung function in patients with CF.

Cystic fibrosis (CF), a genetic disorder caused by mutations of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, is characterised by severe lung disease. While *CFTR* mutations are closely correlated with the pancreatic status, the prognosis of lung, liver and gastrointestinal disease is only marginally predicted from the *CFTR* genotype and might reflect the additional influence of environmental factors and the concurrent expressions of ''modifier'' genes. Likely candidates are genes encoding proteins or enzymes involved in inflammation, immunity, infection, and ion transport.[1]

Nitric oxide (NO) is synthesised from L-arginine by nitric oxide synthases (NOS). All three isoforms (NOS1, NOS2, NOS3) are expressed in the respiratory tract and polymorphisms of *NOS* genes have been described in lung disease. In particular, *NOS1* expression is decreased in epithelial cells from the upper airways of patients with CF.[2] It was proposed that reduced expression of NOS1 could be the cause of the unexpected low or normal exhaled NO concentrations, notwithstanding the presence of chronic inflammation and bacterial colonisation in the airways of CF patients.[3]

The proximal region of the *NOS1* gene contains dinucleotide GT repeats that are located immediately upstream of the transcription start site, next to transcription factor binding sites. Although variants of this region, which exhibits a high heterozygosity index, are not yet fully characterised in humans, gene reporter assays suggest that changes in the structure of *NOS1* 5′-untranslated region (UTR) can markedly affect gene expression and transcription efficiency.[4] In this study we analysed sequence variations of *NOS1* 5′-UTR and sought to determine whether the number of GT repeats affects NOS1 activity, lung NO production, and decline of lung function in patients with CF.

METHODS

Patients

White adult patients with CF and healthy volunteers without recent respiratory tract infections, corticosteroid medication, or tobacco use participated in the study. The diagnosis of CF was based on medical records, repeated sweat chloride tests, and the identification of *CFTR* gene mutations. Patients with one or two *CFTR* mutations of classes 4 or 5 were classified in the ''mild'' *CFTR* genotype group whereas patients with two mutations of classes 1, 2 or 3 were considered as having ''severe'' *CFTR* genotype.[1]

Annual spirometric data (collected from the 5 years before study entry) were used to calculate annual rate of decline (based on a mean of five measurements) of forced expiratory volume in 1 second (FEV_1) in each patient (simple linear regression, accepted R^2 value >0.30).

The study was approved by the local ethical committee and written informed consent was obtained from all subjects.

Exhaled NO measurements

Exhaled NO was measured using a chemiluminescence analyser (NOA^{TM} 280, Sievers, Boulder, CO, USA), sampled in triplicate at a controlled outflow of 100 ml/s, and the mean values recorded according to international guidelines.[5]

NOS1 genotype determination

Genomic DNA extracted from blood mononuclear cells was used for polymerase chain reaction (PCR) amplification (95℃ for 1 minute, 40 cycles of 95℃, 10 s; 67℃, 30 s; 68℃, 12 s; final extension at 68℃ for 2 minutes) with 5-CCTGCGTGGCTACTACATTC-3 (forward) and 5-TGGGTGTGGGGAGGGAGAC-3 (reverse) primers. After purification (Qiaquick PCR purification kit, Qiagen Inc), PCR products were sequenced to determine the number of GT repeats in the polymorphic region. PCR products from homozygous subjects were separated by electrophoresis in 3% agarose gels and used as standards to determine the number of repeats in the PCR products obtained from subsequent individuals.

Statistical analysis

Data were expressed as mean (SD) or percentage with 95% confidence intervals (CI). To assess the combined effect of both alleles on outcome we categorised the alleles above and below the median of GT repeats (27) and the genotypes into three classes (0, 1 or 2 alleles containing more than 27 GT repeats). Mean NO levels and FEV_1 were compared between groups by one way or two way analyses of variance. The Kolmogorov-Smirnov goodness of fit test was used to ascertain that the data were normally distributed before the analysis. Distributions were compared using χ^2 analysis; p values of <0.05 were considered statistically significant.

RESULTS

Fifty nine volunteers (39 men) of mean (SD) age 37.4 (2.3) years and 59 adult patients with CF were studied (table 1). Nineteen *NOS1* alleles were identified, the number of GT repeats in the 5′-UTR region ranging from 18 to 36. *NOS1* genotype distribution was similar in both groups with 24%, 54% and 22% of controls and 27%, 41% and 32% of patients displaying 0, 1, and 2 alleles, respectively, with more than 27 repeats ($\chi^2 = 2.401$, NS).

Exhaled NO values were (plausibly) normally distributed ($Z = 1.284$, NS). Global comparison by one way ANOVA showed a significant association between *NOS1* genotype classes and exhaled NO ($F = 5.078$, $p = 0.008$; fig 1). Two way ANOVA showed that the effect of the number of GT repeats on exhaled NO was independent of the group (controls or patients, $p = 0.003$). Interaction between group and genotype did not reach statistical significance ($p = 0.08$).

Annual decline in FEV_1 was (plausibly) normally distributed among patients ($Z = 1.211$, NS). The retrospective analysis of lung function during the 5 years preceding the study showed different trends according to *NOS1* genotype class ($p = 0.025$) which could not be explained by other clinical parameters (table 1). The annual percentage loss of FEV_1 was 3.3% (95% CI 1.1 to 5.4), 3.2% (95% CI 2.4 to 4.0), and 0.8% (−0.5 to 2.1) for patients with 0, 1, and 2 alleles with >27 repeats, respectively.

DISCUSSION

Over a 5 year period patients with CF displaying more than 27 GT repeats in the 5′-UTR of each *NOS1* allele had a lower annual FEV_1 loss than patients with 27 repeats or less in at least one allele.

Previous studies have indicated that the *NOS1* locus affects lung disease in patients with CF,[6] although the pathophysiological basis for this association remains elusive.

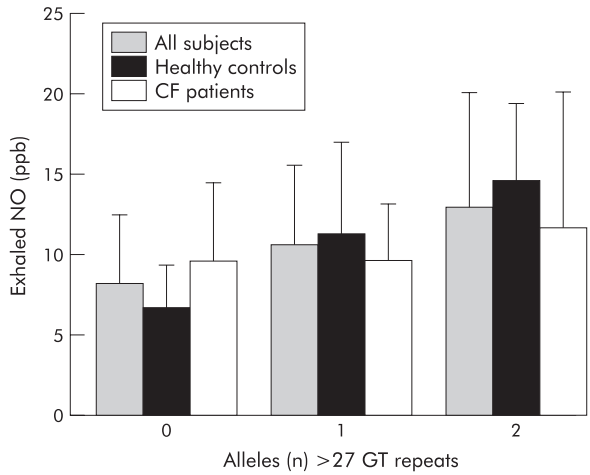

Figure 1 Exhaled NO (in ppb) in controls and patients grouped as a function of the number of alleles containing more than 27 repeats. Values are from left to right: all subjects: 8.1 (95% CI 6.5 to 9.6, n = 30), 10.5 (95% CI 9.1 to 11.8, n = 56), and 12.7 (95% CI 10.1 to 15.2, n = 32) (p = 0.008, ANOVA; healthy controls: 6.4 (95% CI 4.9 to 7.9, n = 14), 11.1 (95% CI 9.1 to 13.2, n = 32), and 14.5 (95% CI 11.8 to 17.1, n = 13); CF patients: 9.5 (95% CI 7.1 to 11.9, n = 16); 9.5 (95% CI 8.0 to 11.0, n = 24), and 11.4 (95% CI 7.5 to 15.3, n = 19).

Expression of NOS1 is dynamically regulated by changes in gene transcription occurring via alternative splicing within the 5′-UTR.[4] Because the GT polymorphism investigated here occurs immediately upstream of the transcription start site of *NOS1*, it might affect the binding affinity of transcription factors[7] which target DNA binding sites located in the vicinity of the repetitive sequence. The number of GT repeats of *NOS1* correlated with exhaled NO concentrations, as reported in previous studies of *NOS1* polymorphism affecting intronic regions.[6 8] Correlation, however, might be partially blunted by factors affecting exhaled NO measurements in CF—for example, altered NO diffusion across the thickened airway mucosal barrier, increased NO consumption by denitrifying bacteria, and combination of NO with reactive oxygen species.[9]

Although CF is a monogenic disease, previous studies have suggested that additional genes could modulate its clinical outcome.[1] Variations in exhaled NO concentrations, associated with an intronic repeat polymorphism of *NOS1*, were found to modulate chronic colonisation of the airway with *Pseudomonas aeruginosa* and *Aspergillus fumigatus*.[6] This

Table 1 Clinical features of CF patients according to *NOS1* genotype

	Number of alleles with more than 27 repeats		
	0	**1**	**2**
Patients (n)	16	24	19
Age (years)	31.4 (8.3)	28.9 (8.3)	28.8 (7.5)
M:F	11:5	11:13	12:7
CFTR genotype (S/M/ND)	13/1/2	18/1/5	12/1/6
Diabetes (n)	4	3	1
Pancreatic insufficiency (n)	15	20	17
BMI (kg/m^2)	19.0 (2.2)	18.9 (2.3)	18.8 (1.7)
Airway bacterial colonisation* (n)	12/1	17/2	18/1
Annual FEV_1 loss (% predicted)			
Mean	3.3	3.2	0.8
95% CI	1.1 to 5.4	2.4 to 4.0	−0.5 to 2.1

CFTR = cystic fibrosis transmembrane regulator; BMI = body mass index; n = number of patients in each category; S, M and ND = severe, mild and not determined *CFTR* genotypes, respectively.
When appropriate, values are expressed as mean (SD). No statistical differences were found (using χ^2 analysis for categories and ANOVA for continuous variables) except for decline in lung function (p = 0.025).
*Colonisation with *P aeruginosa*/*B cepacia*.

association was not found in our study, probably because of the high prevalence of chronic bronchial colonisation in this cohort of adult patients. In addition, except for a decline in lung function, no clinical differences were seen between the three *NOS1* genotype groups (table 1). Other parameters which are not readily accessible to clinical investigation such as transepithelial ion transport, bronchomotor tone, or pulmonary inflammation[10] can vary as a function of NO production, thus affecting the mechanisms controlling lung function decline in CF patients.

Although our results are preliminary and limited by the size of the investigated population, they show an association between the *NOS1* gene locus and progression of lung disease in patients with CF which is independent of the *CFTR* genotype. These findings suggest that *NOS1* variants leading to reduced NO production might be important for understanding the phenotypic disparities of patients with the same *CFTR* mutations. Further investigations are needed to establish the biological consequences of this repeat polymorphism on NOS1 function.

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