Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 361 (2007) 182–188
BBRC
www.elsevier.com/locate/ybbrc

Endothelial nitric oxide synthase gene variants contribute to oxidative stress in COPD

Ehtesham Arif [a,c], Aarif Ahsan [a], Arpana Vibhuti [a], Charu Rajput [a], Desh Deepak [b], Mohammad Athar [c], Bhawani Singh [b], M.A. Qadar Pasha [a,*]

[a] *Functional Genomics Unit, Institute of Genomics and Integrative Biology, Delhi 110 007, India*
[b] *Respiratory Unit, Dr. Ram Manohar Lohia Hospital, New Delhi, India*
[c] *Department of Medical Elementology & Toxicology, Hamdard University, New Delhi, India*

Received 21 June 2007
Available online 16 July 2007

Abstract

Nitric oxide (NO) plays critical role in endothelial dysfunction and oxidative stress in COPD, pointing to the significance of endothelial nitric oxide synthase gene (*eNOS*) variants. We investigated the association of −786T/C, −922A/G, 4B/4A, and 894G/T polymorphisms of *eNOS* with the disease and its impact on nitrite and malonaldehyde levels in 190 COPD patients and 134 healthy controls, all smokers. The −786C, −922G and 4A alleles were significantly over-represented in patients ($p = 0.02$, $p = 0.02$, and $p = 0.03$, respectively). The haplotypes, −786C:4A, 4A:894G, −786C:894G, and −786C:4A:894G were significantly over-represented in patients ($p < 0.0001$, $p = 0.02$, $p = 0.02$, and $p < 0.0001$, respectively), whereas, haplotypes, −786T:4B, 4B:894G, −786T:894G, and −786T:4B:894G were significantly under-represented in the patients ($p < 0.0001$). The patients had significantly increased levels of nitrite ($p = 0.003$) and malonaldehyde ($p < 0.0001$). Combination of genotypes containing −786C and 4A alleles were greater in patients ($p \leqslant 0.05$), and these combinations associated with decreased FEV1 value and nitrite level ($p = 0.03$ and $p = 0.04$, respectively) and with increased malonaldehyde levels ($p = 0.02$). The *eNOS* −786C, −922G, and 4A alleles, these alleles associated haplotypes and genotype combinations were over-represented in patients. The variants and their combinations of four polymorphisms of *eNOS* contribute to disturbed pulmonary function and oxidative stress in COPD.

Keywords: Chronic obstructive pulmonary disease; *eNOS*; Polymorphisms; Nitrite; Malonaldehyde

Chronic Obstructive Pulmonary Disease (COPD) is a common and detrimental disorder with implication for global health [1,2]. Cigarette smoking, although, is the most important risk factor, only 10% of the chronic heavy smokers develop symptomatic COPD suggesting that genetic factors are likely important determinants [2,3]. Observed differences in the course and severity of the disease between racial and ethnic groups together with familial clustering favor a significant hereditary predisposition [4]. Moreover, genetic-environmental interactions possibly account for the underlying mechanisms involved in the pathogenesis of COPD [2,5–7].

The disease is characterized by endothelial dysfunction, inflammation, protease-antiprotease imbalance and oxidative stress [3,8]. Release of oxygen radicals from inflammatory cells, activation and consequent endothelial dysfunction in inflammatory cascade potentiated by both exogenous and endogenous risk factors [3,9–11], aggravate the disease state. Nitric oxide (NO), a multifunctional molecule contributes to the increment of oxidative stress by increasing the peroxinitrite radicals ($ONOO^{\bullet-}$) [11]. The importance of NO has been recognized in respiratory diseases. Increased exhaled NO (FE_{NO}) has been proposed as a marker in lung diseases, especially in case of asthma

Abbreviations: COPD, Chronic obstructive pulmonary disease; NO, Nitric oxide; MDA, Malonaldehyde; eNOS, endothelial nitric oxide synthase gene; VNTR, Variable number of tandem repeats.

* Corresponding author. Fax: +91 11 27667471.
E-mail address: qpasha@igib.res.in (M.A. Qadar Pasha).

0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.07.008

[12] and COPD [13,14]. It is reported that the mechanisms of eNOS enzyme activity and NO bioavailability contribute to the pathophysiology of endothelial dysfunction [9,14,15] and oxidative stress [16]. The ensuing pathophysiological conditions increase the lipid peroxidation (LPO) products in the epithelial lining fluid and urine of COPD patients [10,17]. In the process, lung matrix is damaged leading to emphysema, a major hallmark of COPD [18].

Beside the clinical and biochemical investigations, a great deal of attention is paid to the genetic aspects of COPD by targeting several candidate genes, however, literature is scant on the variants of *eNOS* [5,19], although, the gene seems to be a potential candidate. The 5′-flanking region −786T/C and −922A/G polymorphisms, the 27 base pair 4B/4A repeats of intron 4, and 894G/T polymorphism of exon 7 were associated with reduced nitrite levels in other complex diseases including asthma [5,20–24]. We, thus, envisage that the *eNOS* variants and resultant NO levels along with oxidative stress may play a major role in the pathogenesis of COPD. Our study design based on two objectives, first to ascertain the association of −786T/C, −922A/G, 4B/4A, and 894G/T *eNOS* variants with the disease, their contribution to NO; and second to look for an interaction between the investigated genetic, clinical, and biochemical parameters.

Materials and methods

Study subjects. A total number of 324 north Indian subjects of same ethnicity comprising of 190 COPD (174 males and 16 females) patients and 134 (122 males and 12 females) healthy controls with an age of $\geqslant 40$ years were enrolled at the Respiratory unit, Dr. Ram Manohar Lohia hospital, New Delhi. All the study subjects were smokers and had a history of >10 pack-years. Diagnosis of COPD was based on American Thoracic Society criteria [25]. Spirometry was performed for both controls and patients on Masterscope spirometer 4.2 (Erich Jaeger Laboratories, Wurzburg, Germany). Patients were excluded with evidence of airway obstruction reversibility, which was defined as >200 ml and >15% increase in FEV1 after 30 min of nebulization of 2.5 mg of salbutamol (Asthalin, Cipla, India). Suspected subjects of bronchiectasis and Tuberculosis were excluded based on Chest X-ray followed by high resolution CT scan. All the patients were clinically stable and none had a history of respiratory infection for at least 4-weeks period preceding the study. The study was approved by the Ethics Committee of our Institute and as well as of the hospital. All subjects gave written informed consent to participate in the study.

Biochemical parameters. Plasma nitrite levels were estimated by Griess method (Sigma Co., USA). In a two-step method, the nitrate is first converted to nitrite by nitrate reductase (Sigma Co., USA). The total nitrite is then estimated to reflect NO levels. Samples were estimated in duplicate at 540 nm and the levels, expressed as μmole/liter, were confirmed by repetition where necessary. Inference was drawn from internal standards of nitrite [26]. Malonaldehyde (MDA), a lipid peroxidation (LPO) product, was estimated by a standard method [27]. The biochemical levels were estimated on a high throughput SpectraMax plus 384 Spectrophotometer (Molecular Devices, USA). The coefficients of variations for inter- and intra-batch assessment of total nitrite and MDA levels were <5%.

Genotyping analysis. Genomic DNA was isolated from peripheral blood leukocytes using the modified salting out method [28]. PCR and genotyping of four polymorphisms were carried out by standard protocols [21,22,24]. The −786T/C (rs3918161), −922A/G (rs1800779), and 894G/T (rs1799983) polymorphisms were screened by the Restriction Fragment Length Polymorphism using 8 U of NgoMIV, BslI and BanII restriction endonucleases (New England Bio Labs, Cambridge, UK), respectively, per 20 μl of reaction mixture. The difference in the alleles was resolved by 12% polyacrylamide gel electrophoresis. The 4B/4A, VNTR polymorphism (Ensembl Gene ID-ENSG00000164867), a 27 bp difference, was analyzed on 3% agarose gel electrophoresis. Genotype distribution, linkage disequilibrium (LD), haplotypes, and combinations of genotypes between the two groups were analyzed. Genotypes and haplotypes were used to identify the distribution of specific alleles, whereas, the genotypes and its combinations were used in correlation analyses with FEV1 value, nitrite and MDA levels.

Biostatistical calculations. SPSS 10 and EPIINFO 6 software were used for statistical analysis. Simple Interactive Statistical Analysis software (http://home.clara.net/sisa/) was used for the power calculation. With an alpha value of 0.05 the study provided a power (Two-sided) of >90% for the differences observed between the genotype and allele frequency of −786T/C, −922A/G, and 4B/4A polymorphisms when compared between the two groups. A goodness of fit test was used for testing the Hardy–Weinberg equilibrium and a χ^2 test compared the genotype and allele frequencies of *eNOS* polymorphisms between the two groups. Haplotype frequencies and the extent of association i.e. the Lewontin's coefficient (D') and squared correlation coefficient (r^2) for pairwaise linkage disequilibrium (LD) of the −786T/C, −922A/G, 4B/4A, and 894G/T polymorphisms were calculated by SNP Alyze software (version 3.1; Dynacom, Mobara-shi, Japan). The biochemical parameters were expressed as mean ± SD. Analysis of covariance for adjustment of age, gender and smoking pack-years was carried out by the general linear model procedure to examine the independent effects of biochemical levels/polymorphisms on dependent variables. p values for pairwise differences were corrected for multiple comparisons by Bonferroni correction test. A p value of $\leqslant 0.05$ was considered statistically significant.

Results

Subject characteristics and biochemical measurements

Subject characteristics and biochemical parameters are presented in Supplement Table 1. The COPD patients had significantly lower body mass index (BMI) as compared to controls ($p < 0.0001$). The FEV1, FVC, and FEV1/FVC ratio were expectedly significantly reduced in the patients than controls ($p < 0.0001$, each). The nitrite and MDA levels were significantly increased with a percent difference of 11.09 and 31.05 ($p = 0.003$ and $p < 0.0001$, respectively) in the patients than the controls. p values were adjusted for confounding factors of age, gender, and smoking pack-years.

Genotype distribution and allele frequencies

Results of genotype distribution are presented in Table 1. The two groups were in Hardy–Weinberg equilibrium for −786T/C, −922A/G, 4B/4A, and 894G/T polymorphisms (Supplement Table 2). Genotype distribution for the polymorphisms −786T/C, −922A/G, and 4B/4A differed significantly ($p = 0.02$, $p = 0.02$, and $p = 0.03$, respectively) between the two groups, the homozygotes −786CC, −922GG, and 4A/4A being greater in the patients than controls. Moreover, the genotypes −786TC + CC, −922AG + GG, and 4B4A + 4A4A were also greater in the patients (OR = 1.15; 95% CI = 1.46–3.41; $p = 0.01$,

Table 1
Genotype distribution of −786T/C, −922A/G, 4B/4A, and 894G/T polymorphisms of *eNOS* between controls and patients

Polymorphisms	Genotype distribution		Biostatistics			Allele distribution		Biostatistics	
	Controls, *n* (%)	Patients, *n* (%)	OR (95% CI)	*p*		Controls, *n* (%)	Patients *n* (%)	OR (95% CI)	*p*
786T/C									
−786TT	104 (78%)	117 (62%)	Reference		−786T	236 (88%)	299 (79%)	Reference	
−786TC	28 (21%)	65 (34%)			−786C	32 (12%)	81 (21%)	2.00 (1.28−3.11)	0.004[b]
−786CC	02 (01%)	08 (04%)		0.02[b]					
−786TC + CC[a]	30 (22%)	73 (38%)	1.15 (1.46−3.41)	0.01					
922A/G									
−922AA	104 (78%)	117 (62%)	Reference		−922A	236 (88%)	299 (79%)	Reference	
−922AG	28 (21%)	65 (34%)			−922G	32 (12%)	81 (21%)	2.00 (1.28−3.11)	0.004[b]
−922GG	02 (01%)	08 (04%)		0.02[b]					
−922AG + GG[a]	30 (22%)	73 (38%)	1.15 (1.46−3.41)	0.01					
4B/4A									
4B4B	105 (79%)	120 (63%)	Reference		4B	235 (88%)	297 (78%)	Reference	
4B4A	23 (17%)	57 (30%)			4A	33 (12%)	83 (22%)	1.99 (1.28−3.08)	0.004[b]
4A4A	05 (04%)	13 (07%)		0.03[b]					
4B4A + 4A4A[a]	28 (21%)	70 (37%)	2.55 (1.44−4.51)	0.001					
894G/T									
894GG	95 (71%)	120 (63%)	Reference		894G	223 (83%)	299 (79%)	Reference	
894GT	33 (25%)	59 (31%)			894T	45 (17%)	81 (21%)	1.34 (0.90−2.01)	0.15
894TT	06 (04%)	11 (06%)		0.35					
894GT + TT[a]	39 (29%)	70 (37%)	1.47 (0.862.48)	0.15					

n, number of subjects; OR, odd ratio; CI, confidence interval.
[a] Genotypes were compared with remaining homozygous genotype of the same gene and values are adjusted for the confounding factors such as age, gender, and smoking pack-years.
[b] *p* values were corrected using multiple comparison Bonferroni correction test.

OR = 1.15; 95% CI = 1.46−3.41; $p = 0.01$ and OR = 2.55; 95% CI = 1.44−4.51; $p = 0.001$, respectively). As a consequence, the alleles −786C, −922G, and 4A were over-represented in the patients ($p = 0.004$, $p = 0.004$ and $p = 0.004$, respectively). The distribution of 894G/T polymorphism did not differ between the two groups ($p = 0.35$).

Beside single locus analysis, we also looked for the multilocus associations named as combinations of genotypes, which were effectively used in correlation analysis. The distribution of the possible combinations between any two or all the polymorphisms i.e. −786T/C, 4B/4A, and 894G/T were analyzed. The combinations of genotypes containing −786T, 4B, and 894G alleles viz: −786TT + 4B4B, 4B4B + 894GG, −786TT + 894GG, and −786TT + 4B4B + 894GG were under-represented in patients ($p = 0.02$, $p = 0.01$, $p = 0.1$, and $p = 0.1$, respectively), as a consequence, the combinations of genotypes containing −786C, 4A, and 894T alleles were greater in the patients (Supplement Table 3).

Linkage disequilibrium (LD) analysis

The two polymorphisms −786T/C and −922A/G were in 100% LD ($D' = 1.0$, $r^2 = 1.0$, $p < 0.0001$); −786T/C and 4B/4A polymorphisms were in significant LD in the patients ($D' = 0.4578$, $r^2 = 0.2032$, $\chi^2 = 78.9458$, $p < 0.0001$) and in controls ($D' = 0.22$, $r^2 = 0.0468$, $\chi^2 = 12.0688$, $p < 0.0001$) (Table 2).

Haplotype analysis

Since, −786T/C and −922A/G were in 100% LD, haplotype analyses were performed for the polymorphisms −786T/C, 4B/4A, and 894G/T, only. Maximum likelihood procedure suggested of four haplotypes between any two of −786T/C, 4B/4A, and 894G/T polymorphisms in the two groups (Table 3). The −786C:4A, 4A:894G, and −786C:894G haplotypes were significantly more frequent ($p < 0.0001$, $p = 0.02$, and $p = 0.02$, respectively) and haplotypes −786T:4B, 4B:894G, and −786T:894G were significantly under-represented in the patients ($p = 0.01$, $p < 0.0001$, and $p < 0.0001$, respectively). With respect to the haplotypes among the three polymorphisms, −786T/C, 4B/4A, and 894G/T, total eight haplotypes were possible and the −786C:4A:894G haplotype was significantly higher in the patients ($p < 0.0001$), while −786T:4B:894G haplotype was significantly under-represented in the patients ($p < 0.0001$). The dominance of −786C, −922G, and 4A alleles associated haplotypes was visible in the patients.

Correlation analyses

As can be seen from Table 4, a significant decrease was seen in FEV1 value in the presence of −786TC/−786CC genotype when compared with −786TT genotype in patients as well as controls ($p = 0.02$ and $p = 0.01$, respectively). The same genotypes correlated with significantly

Table 2
Pairwise LD analysis of the four polymorphisms between controls and patients

Pair A	Pair B	Alleles	D'	r^2	χ^2	p
Controls						
−786T/C	−922A/G	268	1.00	1.00	268	<0.0001
−786T/C	4B/4A	268	0.22	0.0468	12.0688	<0.0001
−786T/C	894G/T	268	0.1151	0.000	0.0354	0.8508
4B/4A	894G/T	268	0.1607	0.000	0.0724	0.7879
Patients						
−786T/C	−922A/G	380	1.00	1.00	380	<0.0001
−786T/C	4B/4A	380	0.4578	0.2032	78.9458	<0.0001
−786T/C	894G/T	380	0.0355	0.000	0.0000	0.9352
4B/4A	894G/T	380	0.0736	0.000	0.2632	0.608

D', Lewontin's disequilibrium coefficient; r^2, correlation coefficient.

Table 3
Comparison of haplotypes of −786T/C[a], 4B/4A, and 894G/T polymorphisms between controls and patients

Haplotypes	Control	Patients	χ^2	p[b]	p
−786T:4B	0.7952	0.6913	8.6948	<0.0001	0.01
−786T:4A	0.0854	0.0956	0.197	0.6572	0.686
−786C:4B	0.0817	0.0903	0.1467	0.7017	0.727
−786C:4A	0.0377	0.1228	14.2389	<0.0001	<0.0001
4B:894G	0.7263	0.6116	9.1851	<0.0001	<0.0001
4B:894T	0.1506	0.17	0.4357	0.5092	0.573
4A:894G	0.1058	0.1753	6.0695	0.0138	0.024
4A:894T	0.0174	0.0431	3.3043	0.0691	0.118
−786T:894G	0.7304	0.6175	8.9656	<0.0001	<0.0001
−786T:894T	0.1502	0.1693	0.4235	0.5152	0.577
−786C:894G	0.1017	0.1693	5.9173	0.015	0.025
−786C:894T	0.0177	0.0438	3.3539	0.067	0.155
−786T:4B:894G	0.6615	0.5477	8.4287	<0.0001	<0.0001
−786T:4B:894T	0.1341	0.1423	0.0883	0.7663	0.79
−786C:4A:894G	0.0381	0.1072	10.3588	<0.0001	<0.0001
−786T:4A:894G	0.0684	0.071	0.0163	0.8983	0.906
−786C:4B:894G	0.0642	0.0609	0.0293	0.864	0.888
−786C:4B:894T	0.0171	0.0307	1.1889	0.2755	0.401
−786T:4A:894T	0.0167	0.0258	0.6037	0.4372	0.507
−786C:4A:894T	0.000	0.0144	3.8921	0.0484	0.288

[a] The −922A/G and −786T/C polymorphisms were in 100% LD hence only −786T/C presented.
[b] P values are obtained by permutation test for each haplotype frequency compared between the two groups.

decreased nitrite ($p = 0.05$) and increased MDA levels ($p = 0.04$) in patients, the correlation was marginal in controls ($p = 0.08$ and $p = 0.12$, respectively) (Table 4). The 4B4A/4A4A genotype of the 4B/4A polymorphism and the 894GT/894TT genotype of 894G/T polymorphism correlated with increased MDA levels in patients ($p \leqslant 0.05$), but did not reach significance with FEV1 value and nitrite levels ($p > 0.05$).

Apart from the single locus individual genotypes, multilocus genotypes such as all possible combinations of −786T/C, 4B/4A, and 894G/T polymorphisms were analyzed for their contribution towards FEV1 value, nitrite and MDA levels. The −786TT + 4B4B genotypes combination of the −786T/C and 4B/4A polymorphisms, when compared with the remaining genotypes combinations of the same two polymorphisms, correlated with decreased FEV1 value ($p = 0.03$), nitrite levels ($p = 0.04$) and increased MDA levels ($p = 0.02$) in the patients (Fig. 1), whereas, in controls the combination correlated with only MDA levels ($p = 0.04$). The −786TT + 894GG, 4B4B + 894GG, and −786TT + 4B4B + 894GG genotypes combination were not associated with any of the parameters.

Discussion

The molecular mechanisms that reflect in biochemical parameters in emphysema are inadequately understood. For example, the importance of NO in endothelial dysfunction, inflammation, and oxidative stress has been recognized [9,29], however, surprisingly, *eNOS* has been sparsely investigated for its variants in COPD [5,19]. Although, the functionality of the gene variants have been investigated in other diseases. Between the two promoter region polymorphisms, −786T/C and −922A/G, the former has been identified to reduce NO level, especially with the presence of −786C variant [22]. In case of intron 4 VNTR polymorphism, the 4A allele has been associated with reduced NO levels [30]. The fourth polymorphism 894G/T, under investigation, leads to Glu298Asp change and has been associated with NO levels with conflicting reports [31,32]. Based on the existing evidence and our previous findings [5,19], we hypothesized greater role for these polymorphisms, hence, the present investigation attempted to decipher the role of the *eNOS* variants, resultant nitrite levels, and oxidative stress markers in COPD. It is emphasized that logistic regression analysis was performed to control the influence of potential confounding factors by adjusting genotype data, clinical, and biochemical parameters for age, gender, and smoking pack-years. The two biomarkers nitrite and MDA, studied by us were elevated in patients (Supplement Table 1). Of note, the plasma nitrite levels in the present study and the reported FE_{NO} levels both were elevated in the patients [33]. Importance of NO was visualized in respiratory disease such as asthma since FE_{NO} was suggested as diagnostic test for the patients along with the normal pulmonary function test [12]. Our findings are encouraging as the *eNOS* polymorphisms, −786T/C, −922A/G, and 4B/4A showed an association with the disease. It was interesting to observe that the distribution of −786TC/CC, −922AG/GG, and 4B4A/4A4A genotypes and consequently the frequency of −786C, −922G, and 4A alleles were higher in the patients (Table 2). An earlier study on 4B/4A polymorphism reported that the variants did not differ significantly between COPD patients and controls [19]. Evidence suggests the involvement of the *eNOS*, −786C, −922G, and 4A alleles in cardiopulmonary disorders with endothelial dysfunction as a cardinal pathophysiology [19,21,22,24]. The eNOS enzyme is also reported for its contribution to oxidative stress [16]. Hence, association of *eNOS* variants with the susceptibility

Table 4
Correlation of genotypes of −786T/C*, 4B/4A, and 894G/T polymorphisms with FEV1 (%) value, nitrite and MDA levels in controls and patients

Genotype	FEV1 (%)		Nitrite levels (μmol/L)		MDA levels (μmol/L)	
	Controls	Patients	Controls	Patients	Controls	Patients
−786TT	114.1 ± 20.9 ($n = 104$)	63.7 ± 22.0 ($n = 117$)	84.3 ± 23.1 ($n = 104$)	95.8 ± 26.13 ($n = 17$)	0.45 ± 0.27 ($n = 104$)	0.64 ± 0.26 ($n = 117$)
−786TC + CC[a]	102.7 ± 13.9 ($n = 30$)	55.2 ± 19.7 ($n = 73$)	74.2 ± 18.7 ($n = 30$)	86.1 ± 28.4 ($n = 73$)	0.56 ± 0.24 ($n = 30$)	0.73 ± 0.24 ($n = 73$)
p[b]	0.01	0.02	0.08	0.05	0.12	0.04
4B4B	111.4 ± 20.6 ($n = 105$)	61.7 ± 21.3 ($n = 120$)	81.1 ± 18.2 (n = 105)	91.7 ± 31.1 ($n = 120$)	0.45 ± 0.25 ($n = 105$)	0.65 ± 0.26 ($n = 120$)
4B4A + 4A4A	107.7 ± 14.3 ($n = 28$)	57.0 ± 20.4 ($n = 70$)	78.4 ± 23.7 ($n = 28$)	85.9 ± 22.8 (n = 70)	0.63 ± 0.30 ($n = 28$)	0.74 ± 0.23 ($n = 70$)
p[b]	0.29	0.13	0.51	0.17	0.004	0.05
894GG	108.6 ± 18.2 ($n = 95$)	57.7 ± 20.1 ($n = 120$)	79.9 ± 24.0 ($n = 95$)	91.1 ± 33.2 ($n = 120$)	0.46 ± 0.30 ($n = 95$)	0.63 ± 0.22 ($n = 120$)
894GT + TT	109.6 ± 17.6 ($n = 39$)	61.0 ± 21.6 ($n = 70$)	80.8 ± 22.5 ($n = 39$)	88.4 ± 28.5 ($n = 70$)	0.53 ± 0.33 ($n = 39$)	0.72 ± 0.26 ($n = 70$)
p[b]	0.77	0.29	0.84	0.57	0.23	0.03

n, number of subjects; Values are expressed as mean ± SD.
[a] The −922A/G and −786T/C polymorphisms were in 100% LD hence only −786T/C presented.
[b] p values were corrected using multiple comparison Bonferroni correction test.

to COPD may be reasoned because of the existence of endothelial dysfunction and oxidative stress in the disease.

The importance of single locus associations was further strengthened by the haplotype and LD analyses. Since −786T/C and −922A/G polymorphisms were found in 100% LD (Table 2), further discussion restricted to −786T/C polymorphism only. The haplotype analysis of the −786T/C, 4B/4A and 894A/G polymorphisms reinforced our findings of individual allele's associations. The −786T, 4B and 894G alleles−associated haplotypes such as −786T:4B, 4B:894G, −786T:894G and −786T:4B:894G were significantly under-represented, whereas −786C:4A, 4A:894G, −786C:894G and −786C:4A:894G haplotypes were significantly more frequent in COPD patients (Table 3). The results, therefore, pointed to a possible effect of the *eNOS* variants for an association with COPD. Beside the haplotypes, the under-representation of the combinations of genotypes −786TT + 894GG and 4B4B + 894GG in the patients than controls (Supplement Table 3) further supported the association of *eNOS* polymorphism with COPD.

Apart from the genotype association with the disease, the various correlation analyses between genetic variants and clinical and biochemical parameters showed association with the disease. One of the two notable observations was that the −786TC/CC genotypes correlated with decreased FEV1 values and nitrite levels, and increased MDA levels in the patients (Table 4). Furthermore, the combinations of genotypes containing −786C and 4A alleles, also, correlated with reduced FEV1 values and nitrite levels and increased MDA levels (Fig. 1). Although, it seems ours is the first report on the association of *eNOS*

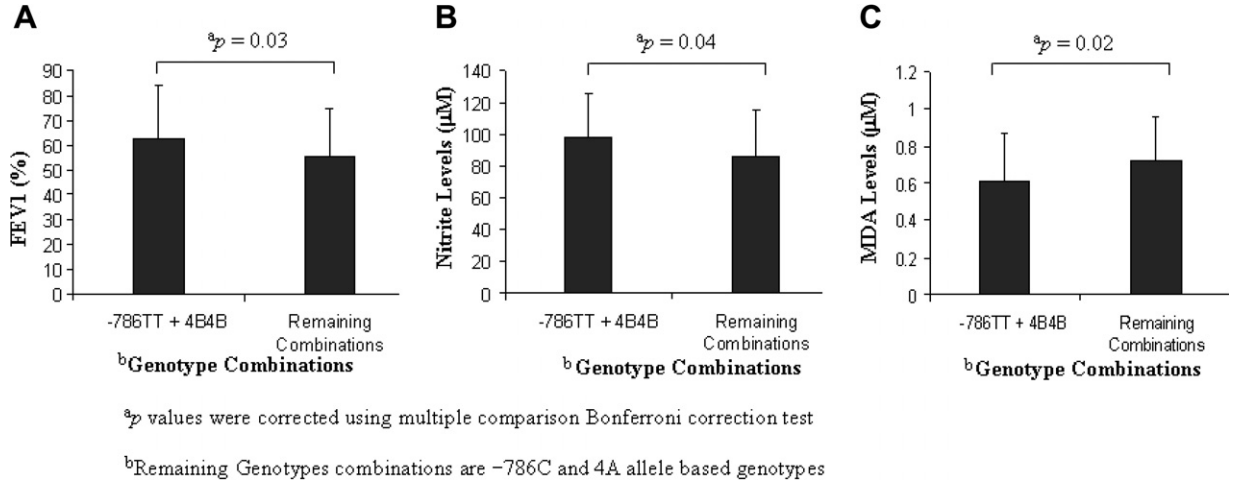

Fig. 1. (A) Correlation of genotypes combinations of −786T/C + 4B/4A polymorphism and remaining combination with FEV1 (%) value in patients. (B) Correlation of genotypes combinations of −786T/C + 4B/4A polymorphism and remaining combination with Nitrite levels in patients. (C) Correlation of genotypes combinations of −786T/C + 4B/4A polymorphism and remaining combination with MDA levels in patients.

polymorphisms with FEV1 value, nitrite, and MDA levels in COPD, however, these variants have also been correlated with decreased nitrite levels in context of other diseases [20,22,23,30]. In relation to respiratory diseases, the 894G/T polymorphism has been investigated in asthma and the G allele associated genotype was shown to correlate with increased FE_{NO} [23]. Interestingly, in our study, although, −786T/C and 4B/4A variants corresponded to decreased nitrite levels (Table 4 and Fig. 1B), the overall nitrite levels were found to be increased in patients as compared to the controls (Supplement Table 1), which could be attributed to other factors. We envisage, the *eNOS* containing −786C, −922G, and 4A alleles becomes faulty resulting in reduced NO generation (Table 4 and Fig. 1B). However, as a compensatory mechanism, the decrement of NO in endothelium may stimulate the inducible NOS (iNOS), which can also get activated by inflammation. The explanation is in agreement with earlier findings, wherein augmented NO levels in altered eNOS conditions were associated with upregulation of iNOS expression in the lungs [34,35]. Under the pathophysiological conditions more of the NO combines with $O_2^{\cdot -}$ to produce peroxinitrite radical [36]. The increased peroxinitrite, in combination with $OH^{\cdot}$ may result in increased LPO, which we, in fact, observed in the patients (Supplement Table 1).

To conclude over-representation of −786C, −922G, and 4A alleles, elevated nitrite and MDA levels were observed in COPD patients. These genetic variants directly or indirectly contribute to the increment of oxidative stress by altering the expression or activity. Of note, these variants also correlated with reduced FEV1 values thus abetting the disease. It may necessitate establishing the NO production both at eNOS and iNOS levels. As the findings are novel, they deserve to be tested in larger such study subjects.

Acknowledgments

CSIR, India, supported this work under the Toxicogenomics Task force (CMM–0003). We highly appreciate Professor Samir K. Brahmachari, Director, IGIB for his constant encouragement.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.07.008.

References

[1] S.S. Hurd, International efforts directed at attacking the problem of COPD, Chest 117 (2000) 336S–338S.
[2] G. Snider, Chronic obstructive pulmonary disease: risk factors, pathophysiology and pathogenesis, Annu. Rev. Med. 40 (1989) 411–429.
[3] P.J. Barnes, Chronic obstructive pulmonary disease, N. Engl. J. Med. 343 (2000) 269–280.
[4] B.H. Cohen, E.L. Diamond, C.G. Graves, K. Kreiss, D.A. Levy, H.A. Menkes, S. Permutt, S. Quaskey, M.S. Tockman, A common familial component in lung cancer and chronic obstructive pulmonary disease, Lancet 2 (1977) 523–526.
[5] A. Ahsan, R. Ram, M.A. Baig, M.A. Qadar Pasha, ACE I allele and eNOS G allele crosstalk may have a role in chronic obstructive pulmonary disease, Clin. Biochem. 37 (2004) 1037–1040.
[6] A.J. Sandford, E.K. Silverman, Chronic obstructive pulmonary disease. 1: susceptibility factors for COPD the genotype-environment interaction, Thorax 57 (2002) 736–741.
[7] E. Arif, A. Vibhuti, P. Alam, D. Deepak, B. Singh, M. Athar, M.A. Qadar Pasha, Association of CYP2E1 and NAT2 gene polymorphisms with chronic obstructive pulmonary disease, Clin. Chem. Acta. 382 (2007) 37–42.
[8] V.I. Peinado, J.A. Barbera, J. Ramirez, F.P. Gomez, J. Roca, L. Jover, J.M. Gimferrer, R. Rodriguez-Roisin, Endothelial dysfunction in pulmonary arteries of patients with mild COPD, Am. J. Physiol. 274 (1998) L908–L913.
[9] J.F. Arnal, A.T. Dinh-Xuan, M. Pueyo, B. Darblade, J. Rami, Endothelium-derived nitric oxide and vascular physiology and pathology, Cell Mol. Life Sci. 55 (1996) 1078–1087.
[10] W. MacNee, I. Rahman, Oxidants and antioxidants as therapeutic targets in COPD, Am. J. Respir. Crit. Care. Med. 160 (1999) S58–S65.
[11] C.M. Sanguinetti, Oxidant/antioxidant imbalance: role in the pathogenesis of COPD, Respiration 59 (1992) 20–23.
[12] A. Deykin, A.F. Massaro, J.M. Drazen, E. Israel, Exhaled nitric oxide as a diagnostic test for asthma: online versus offline techniques and effect of flow rate, Am. J. Respir. Crit. Care. Med. 165 (2002) 1597–1601.
[13] G. Adamkiewicz, S. Ebelt, M. Syring, J. Slater, F.E. Speizer, J. Schwartz, H. Suh, D.R. Gold, Association between air pollution exposure and exhaled nitric oxide in an elderly population, Thorax 59 (2004) 204–209.
[14] M. Corradi, P. Montuschi, L.E. Donnelly, A. Pesci, S.A. Kharitonov, P.J. Barnes, Increased nitrosothiols in exhaled breath condensate in inflammatory airway diseases, Am. J. Respir. Crit. Care. Med. 163 (2001) 854–858.
[15] Y. Miyamoto, Y. Saito, M. Nakayama, Y. Shimasaki, T. Yoshimura, M. Yoshimura, M. Harada, N. Kajiyama, I. Kishimoto, K. Kuwahara, J. Hino, E. Ogawa, I. Hamanaka, S. Kamitani, N. Takahashi, R. Kawakami, K. Kangawa, H. Yasue, K. Nakao, Replication protein A1 reduces transcription of the endothelial nitric oxide synthase gene containing a –786T/C mutation associated with coronary spastic angina, Hum. Mol. Genet. 9 (2000) 2629–2637.
[16] U. Forstermann, T. Munzel, Endothelial nitric oxide synthase in vascular disease: from marvel to menace, Circulation 113 (2006) 1708–1714.
[17] V.L. Kinnula, Focus on antioxidant enzymes and antioxidant strategies in smoking related airway diseases, Thorax 60 (2005) 693–700.
[18] A. Cantin, R.G. Crystal, Oxidants, antioxidants and the pathogenesis of emphysema, Eur. J. Respir. Dis. 66 (1985) 7–17.
[19] P. Yildiz, H. Oflaz, N. Cine, N. Erginel-Unaltuna, F. Erzengin, V. Yilmaz, Gene polymorphisms of endothelial nitric oxide synthase enzyme associated with pulmonary hypertension in patients with COPD, Respir. Med. 97 (2003) 1282–1288.
[20] A. Ahsan, T. Norboo, M.A. Baig, M.A. Qadar Pasha, Simultaneous selection of wild-type genotypes of G894T and 4B/4A polymorphisms of NOS3 gene associates with HA adaptation, Ann. Hum. Genet. 69 (2005) 260–267.
[21] K. Hibi, T. Ishigami, K. Tamura, S. Mizushima, N. Nyui, T. Fujita, H. Ochiai, M. Kosuge, Y. Watanabe, Y. Yoshii, M. Kihara, K. Kimura, M. Ishii, S. Umemura, Endothelial nitric oxide gene polymorphism and acute myocardial infarction, Hypertension 32 (1998) 521–526.
[22] M. Nakayama, H. Yasue, M. Yoshimura, Y. Shimasaki, K. Kugiyama, H. Ogawa, T. Motoyama, Y. Saito, Y. Ogawa, Y. Miyamoto, K. Nakao, 786T/C mutation in the 5′-flanking region of the endothelial nitric oxide synthase gene is associated with coronary spasm, Circulation 99 (1999) 2864–2870.

[23] K. Storm van's Gravesande, M.E. Wechsler, H. Grasemann, E.S. Silverman, L. Le, L.J. Palmer, J.M. Drazen, Association of a missense mutation in the NOS3 gene with exhaled nitric oxide levels, Am. J. Respir. Crit. Care. Med. 168 (2003) 228–231.
[24] X.L. Wang, A.S. Sim, R.F. Badenhop, R.M. McCredie, D.E. Wilcken, A smoking dependent risk of coronary artery disease associated with a polymorphism of the endothelial nitric oxide synthase gene, Nat. Med. 2 (1996) 41–45.
[25] American Thoracic Society. Standardization of Spirometry. Am. Rev. Respir. Dis.136 (1987) 1285–298.
[26] L.C. Green, D.A. Wagner, J. Glogowski, P.L. Skipper, J.S. Wishnok, S.R. Tannenbaum, Analysis of nitrate, nitrite and [^{15} N] nitrate in biological fluids, Anal. Biochem. 126 (1982) 131–138.
[27] J.R. Wright, H.D. Colby, P.R. Miles, Cytosolic factors which affect microsomal lipid peroxidation in lung and liver, Arch. Biochem. Biophys. 206 (1981) 296–304.
[28] S.A. Miller, D.D. Dykes, H.F. Polesky, A simple salting out procedure for extracting DNA from human nucleated cells, Nuc. Acids. Res. 16 (1988) 1215.
[29] R. Schulz, T. Rassaf, P.B. Massion, M. Kelm, J.L. Balligand, Recent advances in the understanding of the role of nitric oxide in cardiovascular homeostasis, Pharmacol. Ther. 108 (2005) 225–256.
[30] T. Tsukada, K. Yokoyama, T. Arai, F. Takemoto, S. Hara, A. Yamada, Y. Kawaguchi, T. Hosoya, J. Igari, Evidence of association of the ecNOS gene polymorphism with plasma NO metabolite levels in humans, Biochem. Biophys. Res. Commun. 245 (1998) 190–193.
[31] B.A. Veldman, W. Spiering, P.A. Doevendans, G. Vervoort, A.A. Kroon, P.W. de Leeuw, P. Smits, The Glu298Asp polymorphism of the NOS3 gene as a determinant of the baseline production of nitric oxide, J. Hypertens. 20 (2002) 2023–2027.
[32] Y. Yoon, J. Song, S.H. Hong, J.Q. Kim, Plasma nitric oxide concentrations and nitric oxide synthase gene polymorphisms in coronary artery disease, Clin. Chem. 46 (2000) 1626–1630.
[33] K. Ansarin, J.M. Chatkin, I.M. Ferreira, C.A. Gutierrez, N. Zamel, K.R. Chapman, Exhaled nitric oxide in chronic obstructive pulmonary disease: relationship to pulmonary function, Eur. Respir. J. 17 (2001) 934–938.
[34] L. Connelly, A.T. Jacobs, M. Palacios-Callender, S. Moncada, A.J. Hobbs, Macrophage endothelial nitric-oxide synthase autoregulates cellular activation and pro-inflammatory protein expression, J. Biol. Chem. 278 (2003) 26480–26487.
[35] S. Cook, P. Vollenweider, B. Menard, M. Egli, P. Nicod, U. Scherrer, Increased eNOS and pulmonary iNOS expression in eNOS null mice, Eur. Respir. J. 21 (2003) 770–773.
[36] Y.M. Xie, M.S. Wolin, Role of nitric oxide and its interaction with superoxide in the suppression of cardiac muscle mitochondrial respiration. Involvement in response to hypoxia/reoxygenation, Circulation 94 (1996) 2580–2586.