Positive association between two polymorphic sites (+134 insA/delA and G198T) of the endothelin-1 gene and chronic obstructive pulmonary disease. A case-control study

Fotis Sampsonas a, Anna Antonacopoulou b, Dionysios Spathas c, Dimosthenis Lykouras a, Haralabos Kalofonos d, Christodoulos Flordellis e, Kostas Spiropoulos a,*, Nikolaos Siafakas f

a University Hospital of Patras, Department of Pulmonology, 26500 Patras, Greece
b University of Patras, Medical School, Clinical Oncology Laboratory, 26500 Patras, Greece
c University of Patras, Medical School, Laboratory of General Biology 26500 Patras, Greece
d University of Patras, Medical School, Department of Internal Medicine, Division of Oncology and Clinical Oncology Laboratory, 26500 Patras, Greece
e University of Patras, Medical School, Department of Pharmacology, 26500 Patras, Greece
f University of Crete, Medical School, Department of Thoracic Medicine, 71110, Heraklion Crete, Greece

Received 28 September 2008; accepted 15 June 2009
Available online 28 July 2009

KEYWORDS
COPD;
Polymorphisms;
Endothelin-1;
Inflammation

Summary
Endothelin-1 (ET-1) has been implicated in the pathogenesis of Chronic Obstructive Pulmonary Disease (COPD) for establishing an inflammatory loop in the respiratory mucosa that could become independent from the initial irritant factor. Common causes of COPD exacerbations are associated with elevated ET-1 sputum concentrations. Genetic variants of the ET-1 gene, that lead to elevated ET-1 peptide levels, have not been investigated in COPD.

We performed a case control, genetic study to assess possible associations of two polymorphisms of the ET-1 gene, an adenine insertion (+134 insA/delA) and a guanine to thymine transversion (G198T) with the COPD phenotype and disease severity.

The genotypes of 209 subjects, 107 COPD smokers (patients) and 102 non-COPD smokers (controls) were examined. Statistical analysis revealed that the 3A/4A and 4A/4A genotypes were more common (P < 0.01) in patients. Moreover, a protective effect against COPD of the TT genotype (G198T) was exhibited. COPD smokers were carrying more frequently the GG genotype and less frequently the TT genotype (P = 0.047). Diplotypic analysis revealed that subjects carrying the 3A3A;TT genotype had a lower risk of COPD development (P = 0.027).
Introduction

Chronic Obstructive Pulmonary Disease (COPD) is a widespread multifactorial disease with a heterogeneous pathogenesis. Cigarette smoking is the major risk factor, however only 10–20% of smokers develop symptomatic COPD. This, along with several other lines of evidence, such as, familial clustering, twin studies, accelerated decline of Forced Expiratory Volume in 1 s (FEV1), suggest complex genetic components for the disease. A-1 antitrypsin deficiency is the only proven genetic risk factor for COPD development, while numerous association studies examine other candidate gene loci to establish involvement in COPD. Failure to replicate some of these studies makes them controversial. COPD is the fourth leading cause of morbidity and mortality, expected to rise to the third position, by the year 2020.

Recent studies revealed that in COPD, response to inflammation, especially in small airways, is characterized by an amplification that does not cease when cigarette smoking is withdrawn, thus becoming independent of the causal mechanism. It has been reported that high levels of Endothelin-1 (ET-1) in BronchoAlveolar Lavage (BAL) are implicated in COPD pathogenesis, via their involvement in the inflammatory cycle. ET-1 preserves inflammation in the respiratory mucosa independently of the initial inflammatory stimulus, therefore establishing an autocrine–paracrine loop.

Endothelin-1 is an important regulator of inflammation in the lung milieu and is produced by vascular endothelial cells, bronchial epithelial cells, pulmonary monocytes, polymorphonuclear leukocytes and fibroblasts.

An explanation of the COPD pathogenesis and pulmonary hypertension development proposes apoptosis of alveolar and epithelial cells followed by the failure of vascular epithelial repair as a cause of emphysema. Smoke exposure provokes many harmful vascular effects, such as increasing the expression of vasoconstrictor and mitogenic factors, like ET-1. The “earliest” pathology of the lungs of smokers is the development of intimal thickening of pulmonary arteries, the severity of which is correlated with the daily number of cigarettes smoked. Therefore, the autocrine actions of ET-1 (pro-inflammatory, vasoconstrictor and mitogenic) may be involved even in early lung remodeling due to smoking, and also could contribute to disease severity and pulmonary hypertension in COPD, since failure of alveolar and epithelial repair is a characteristic of advanced disease.

The human ET-1 gene is located on chromosome 6p and consists of 5 short exons and 4 introns. Its product is synthesized as a precursor molecule, the proendothelin that is hydrolyzed by many converting enzymes, to the mature active 21 amino acid ET-1 peptide. Lung displays the highest levels of ET-1 production and activity among all other organs maintained by activation of gene transcription, since precursor or active ET-1 peptides are not stored within cells. Lung is also the main site of ET-1 clearance from circulation. ET-1 acts in the lung tissue as an autocrine–paracrine signal on ET-A and ET-B receptors that mediate its activities locally, while a small portion of the total ET-1 production is secreted in the blood, where its half life time is 4–7 min.

Single-nucleotide polymorphisms (SNPs) of ET-1 gene have been associated with variations in ET-1 levels. An adenine insertion SNP is located 138 bp downstream of the transcription start site in the 5′-UTR (Untranslated Region), in exon 1 (insA/delA). Transfection studies using reporter constructs showed that this insertion could account for elevated ET-1 levels, probably due to increased mRNA stability, rather than translation efficacy. It is possible that preproET-1 mRNA forms different stem-loops of 5′-UTR transcripts and that adenine insertion changes the free energy, number and secondary structure of stem-loops, thus affecting transcriptional stability.

A single-nucleotide polymorphism in exon 5, which replaces guanine by thymine (G198T, G→T transversion Codon 198) leading to substitution of a lysine by asparagine (Lys198Asn), has also been associated with elevated ET-1 blood levels. As far as polarity and charge are concerned lysine and asparagine belong to different amino acid groups, therefore substitution of lysine by asparagine may alter the tertiary structure of the protein and consequently its function (stability and protein–protein interaction).

Since ET-1 is involved in the maintenance of inflammation in the lung of COPD patients, it is plausible that polymorphisms of the ET-1 gene, which affect availability and function of its molecule, may modulate the risk of COPD development. To test this hypothesis, we conducted a case-control study to examine the differences in the frequency of these SNPs among COPD smokers (patients) and non-COPD smokers (controls) as well as their effects on lung function in a Caucasian Greek population.

Methods

Subjects

The study cohort consisted of 209 subjects, including 107 consecutive COPD smokers, defined as patients, that were recruited from the outpatient pulmonary clinic of Patras’ University Hospital from March 2005 to January 2007 and 102 non-COPD smokers, defined as controls, randomly selected from the general population (people visiting the hospital for a health check-up and community volunteers) and overall matched to the group of patients for age.
smoking habit (pack-years), Body Mass Index (BMI, kg/m²) and sex. All participants were current or ex-smokers with a history of at least 20 pack years of smoking and were subjected to spirometry, including a bronchodilator test, using a computerized system, by the same technician (Pulmolab 435 Morgan Data Acquisition System 401, USA) according to established guidelines. Patients with poorly reversible airflow limitation associated with bronchiectasis, cystic fibrosis and fibrosis due to tuberculosis were excluded. The cumulative cigarette dose (pack-year) was calculated using the following formula: pack-year = (packs per day) \( \times \) (years of smoking).

Regarding the Forced Expiratory Volume in 1 s (FEV₁), the higher of the values obtained in two technically satisfactory tracings was taken. Additionally, FEV₁ reversibility after inhalation of 200 μg salbutamol was <12% of pre-bronchodilator FEV₁. A flow volume loop was also obtained for all subjects and the Forced Expiratory Flow of the 25%, 50%, 75% of the Forced Vital Capacity (FEF₂₅, FEF₅₀, FEF₇₅, respectively) and the average expiratory flow over the middle half of the FVC (FEF₂₅–₇₅) were recorded.

Patients satisfied the criteria proposed by the Global Initiative for Chronic Obstructive Pulmonary Disease [GOLD]. GOLD scales of severity were used to verify stages of the disease. Non-COPD smokers (control group) had normal spirometry and no respiratory symptoms. All subjects had no major comorbidities such as heart failure, renal dysfunction, cancer or severe hypertension.

The study was approved by the University of Patras Ethics Committee and the Scientific Committee of the University Hospital of Patras, and all subjects signed a patient’s consent form.

Blood collection and genotyping

DNA was isolated from 3 ml of whole blood, using QIAamp DNA blood mini kit (QIAGEN). The ET-1 gene polymorphisms +134 insA/delA in the 5’ untranslated region and G198T, in exon 5, were genotyped in 107 patients and 102 controls. Genotyping was performed with real time PCR using the MX3000p (Stratagene, La Jolla, CA, USA). The primers and MGB Taqman probes used for the G198T polymorphism were as previously reported. For the +134 insA/delA polymorphism the primers 5'TTC TCT CCT GGC AGG-3' and 5'-ATC TCA AAG CGA TCC TTC-3' were used in conjunction with the LNA (Locked Nucleic Acid) Taqman probes 5'-(6-Fam) AG + TGCC + C + T + T + TAACGG (BHq1)-3' (for 3A allele) and 5'-(Hex) AGT GCC + C + T + T + T + TAA + CG + GG (BHq1)-3' (for 4A allele) where a '*' before the base indicates an LNA base. Primers were synthesized by Metabion International (Martinsried, Germany), MGB probes by Applied Biosystems (Foster City, CA, USA) and LNA probes by Sigma–Prolog (The Woodlands, TX, USA). Reactions were performed using Brilliant QPCR Master Mix (Stratagene).

Statistical analysis and methodology

The normality of the numerical parameters was tested using the Kolmogorov–Smirnov test. Comparisons of demographic data between groups were performed with the Chi-squared analysis or unpaired t-test, as appropriate. Logistic regression analysis was used to quantify the association between the genotypes and COPD, since it is applicable for broader range of research situations, compared to discriminated analysis. Chi-squared analysis was used for categorical variables. Correlations of spirometric values (FEV₁, FEF₂₅–₇₅) with genotypes were tested with one-way ANOVA for normally distributed variables (within the patient and control groups) and Kruskal-Wallis and Mann-Whitney tests for non-normally distributed variables. For multiple comparisons, the ANOVA tests were followed by a post hoc Bonferroni test. P-values lower than 0.05 were considered significant. Statistical analysis was performed using SPSS statistical package (SPSS, Release 14.0.1, Chicago, IL, USA). Haplotypic analysis was performed using the FAMHAP software program, based on the Monte-Carlo simulations. The haplotype frequencies computed by the program are maximum-likelihood estimates (MLEs), obtained with the expectation-maximization (EM) algorithm (http://famhap.meb.uni-bonn.de).

Results

Characterization of subjects

The baseline characteristics of the 209 subjects, representing a homogeneous Greek population, are listed in Table 1. The parameters of FEV₁ and FEV₁/FVC were significantly decreased in the COPD patients compared to controls. The group of COPD patients consisted of 99 active and 8 ex-smokers, whereas the control group consisted of 95 active and 7 ex-smokers.

Genotypes of the control group were found by Pearson’s goodness-of-fit Chi-square test to be in Hardy–Weinberg equilibrium (P = 0.21 for the +138 3A/4A SNP and P = 0.17 for the G198T). Table 2 summarizes the distribution of genotypes (of the adenine insertion/deletion +134 insA/delA) and G198T SNPs in patients and controls. Statistical analysis revealed that the frequencies of the +134ins/delA genotype was statistically significantly different between patients and controls (P = 0.017, \( x^2 = 8.178 \)). Subjects carrying the 3A4A and the 4A4A genotypes had increased risk of COPD development (OR = 1.427, 95%CI = 1.089–1.871 and OR = 2.622, 95%CI = 0.842–8.165, respectively, Reference genotype:3A3A).

Regarding the G198T SNP, 69 out of 107 COPD smokers carried the GG genotype, 34 the GT genotype and 4 the TT genotype. Among controls, 53 out of 102 subjects carried the GG genotype, 37 the GT genotype and 12 the TT genotype. The distribution of these genotype was significantly different between patients and controls (P = 0.047, \( x^2 = 6.109 \) (Table 2). Statistical analysis revealed that subjects carrying the GG genotype were in increased risk of COPD development (OR = 1.241, 95%CI = 0.982–1.568, Reference genotype:GT/TT) (Table 2).

Distribution of the 3A, 4A alleles revealed that the 3A allele is more common in controls, whereas the 4A allele was significantly more common in patients (\( x^2 = 6.42, P = 0.011 \)). Regarding the 198G, T alleles statistical analysis revealed that the G allele was more common in patients,
whereas the T allele was more common in controls ($\chi^2 = 5.94, P = 0.014$).

Within the total study cohort, patients carrying the 3A3A genotype had higher FEV$_1$ and FEF$_{25-75}$ values compared to 3A4A ($P < 0.05$-Bonferroni analysis for FEV$_1$ and FEF$_{25-75}$) and 4A4A genotypes ($P < 0.05$ for FEV$_1$), whereas subjects carrying the TT genotype had higher FEV$_1$ and FEF$_{25-75}$ values compared to those carrying the GG genotype ($P < 0.005$).

Within the COPD group, carriers of the TT genotype (G198T SNP) demonstrated statistically significant higher FEV$_1$ ($P = 0.014$-Bonferroni analysis) and FEF$_{25-75}$ ($P = 0.006$-Bonferroni analysis) spirometric values (Fig. 1a and b respectively). Statistical analysis revealed that carriers of the GT genotype had more often mild or moderate disease (GOLD I–II) ($P = 0.004$, OR = 3.562, 95%CI = 1.505–8.430). Moreover, carriers of at least one T allele (GT or TT genotypes) had most probably GOLD I–II compared to patients carrying the GG genotype ($P = 0.004$, OR = 3.385, 95%CI = 1.475–7.768). No significant correlations of the +134 SNP, in patient group, with disease severity and spirometric values were noted (Fig. 2a,b).

Analysis of both genotypes in combination revealed that carriers of the 3A3A; GG genotype were less likely to develop mild-moderate disease ($P = 0.006$, OR = 0.164, 95%CI = 0.044–0.602).

All theoretically possible haplotypes exist in the examined population. Haplotypic distribution of genotypes was remarkably different among patients and controls (Global $P = 0.0011$ (Table 3a), revealing that subjects with 4A:T and 4A:G haplotypes were in increased risk of COPD development (OR = 1.725, 95%CI = 0.4209–7.0828, and OR = 1.589, 95%CI = 0.844–3.0064, respectively) while subjects with 3A:T haplotype had a lower risk in COPD development (OR = 0.442, 95%CI = 0.219–0.9212), indicating the important role of the 4A allele in disease development (Table 3a).

Additionally, haplotypic distribution was also diverse among patients with less (GOLD I–II) or more severe (GOLD III–IV) disease (Global $P = 0.0165$ (Table 3b)). Subjects with the 3A:G haplotype were in increased risk of severe COPD development (GOLD III–IV) (OR:1.8162, 95%CI = 1.0367–3.1819), whereas subjects carrying the 3A:T and 4A:T haplotypes had lower risk of severe COPD development (OR = 0.4812, 95%CI = 0.2145–1.0793 and OR = 0.3209, 95%CI = 0.0744–1.3842, respectively), revealing the significant role of G allele in the disease severity (Table 3b).
associated with elevated sputum ET-1 concentrations. 

Additionally the pro-inflammatory cytokines, IL-6 and IL-8, are elevated in the airways of COPD patients both during and in-between exacerbations. An interesting positive feedback loop between ET-1 peptide and IL-6 and IL-8 has been described in lung epithelial cells. Once the loop is initiated, it may become independent of the original inflammatory stimulus. Inflammation in COPD persists for a long time, even years, after smoking cessation. Acute smoke exposure induces the expression of the ET-1 peptide which might maintain inflammation after withdrawal of smoking.

Additionally, ET-1 is an important local mediator of inflammation in the lung. ET-1 is a powerful chemo-attractant for various inflammatory cells, especially eosinophils. Patients with COPD that do not currently smoke have increased number of inflammatory cells, i.e. neutrophils, macrophages, and astonishingly eosinophils, in their sputum. Furthermore, ET-1 promotes mucus secretion, airway smooth muscle constriction, microvascular leakage, vascular cell adhesion molecules and matrix metalloprotease secretion all of which are implicated in COPD pathogenesis.

It’s plausible that increased ET-1 levels due to polymorphisms in ET-1 gene might result in enhanced inflammatory response to common environmental stimuli (i.e. cigarette smoking, viral infection) that may eventually become independent from the initial stimuli. Recent studies highlighted the fact that SNPs in the ET-1 gene are implicated in the elevation of ET-1, probably due to increased stability of the ET-1 mRNA. Therefore, carriers of such genetic alterations could face an increased risk of COPD development in response to environmental stimuli, as a result of increased ET-1 levels, compared to non-carriers.

In the present study we focused our attention on two biologically and probably clinically significant polymorphic sites of the ET-1 gene. We have demonstrated that the adenine insertion SNP is associated with the COPD

Figure 1  Comparison of FEV1 (a) and FEF 25–75 (b) values and their associations with G198T SNP (GG, GT, TT genotypes), in the patient group.

Figure 2  Comparison of FEV1 (a) and FEF 25–75 (b) values and their associations with +134insA/delA SNP (3A3A, 3A4A, 4A4A genotypes), in the patient group.
phenotype and lower FEV1 values and therefore the +138 4A allele is implicated in COPD pathogenesis. It is possible that subjects carrying at least one 4A allele create a pre-proET-1 mRNA that forms different stem-loops of 5’-UTR transcripts and that adenine insertion changes the free energy, number and secondary structure of stem-loops. Further studies are needed to elucidate the mechanisms underlying these associations. However, we tempt to speculate that this polymorphism might result in increased production of ET-1 in response to cigarette smoke, self-preservation of inflammation and finally COPD development. Haplotypic analysis revealed that the 4A:G and 4A:T haplotypes are associated to COPD development, confirming the aforementioned data for the involvement of the 4A allele in disease progression. Nevertheless, the 4A allele was not related to disease severity, but may be involved in early lung remodeling due to smoking.14

Regarding the G198T SNP, our study revealed that the TT genotype was more prevalent among non-COPD smokers (controls) and that the T allele was related to better lung function tests and milder COPD. On the other hand, the G allele, the GG genotype and the G: 3A haplotype are related to severe (GOLD III–IV) COPD. The mechanism by which the T allele seems to confer protection against COPD development, while the G allele is associated with disease severity is not clear. This SNP hasn’t been studied in relation to ET-1 peptide levels in lung tissue. Moreover, even though the G198T SNP is responsible for an amino acid change (Lys198Asn), the position of this SNP is not in proximity with the regulatory loci of the transcriptional sites.30 Thus, it is rather improbable to modulate the precursor ET-1 molecules. This does not exclude the possibility that the SNP is in linkage disequilibrium with the actual protecting genetic locus, since it is in proximity with the HLA group of antigens and TNF group of genes that are implicated in inflammatory process and tissue damage. Moreover, the amino acid substitution might decrease the activity of ET-1 (stability and protein-protein interaction) or interfere with the cleavage of the peptide. Lysine and asparagine belong to different amino acid groups (with diverse polarity and charge), therefore substitution of lysine by asparagine may alter the tertiary structure of the protein.

Since the first reference for peptidergic activity produced in endothelial cells31 ET-1 was put forward as a promising molecule implicated in numerous pathogenic pathways. Our study is, to our knowledge, the first to associate two ET-1 polymorphisms with the COPD phenotype. Our results indicate that the +138 4A/3A and G198T SNPs are significantly involved in COPD pathogenesis and therefore prove useful as markers for the identification of subjects at increased risk to COPD development. Further studies will clarify the role of these polymorphisms in COPD development and the molecular pathways underlying their action.

Acknowledgements

Dr. Sampsonas is financially supported by Grants of the Hellenic Thoracic Society for this work.

Authors’ contributions

FS carried out the experimental work and wrote the manuscript. DL produced the illustrations. AA carried out the molecular modelling studies. KS conceived the hypothesis, supervised the experimental work and assisted in drafting the manuscript. CF advised on experimental work. NS assisted in drafting the manuscript. All authors read and approved the final manuscript.

Conflict of interest

None of the authors have conflict of interest to declare in relation to this work.

References


