Original Article

Klotho gene polymorphism -395 G<A in patients with chronic obstructive pulmonary disease (COPD)

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Key words:
- *Klotho* gene
- COPD
- BMI
- emphysema

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SUMMARY. Background: The function of the *Klotho* gene, originally identified by insertional mutagenesis in mice, is to suppress multiple aging phenotypes. It has been shown that a mutant *Klotho* gene is associated with pulmonary emphysema in mice. The aims of this study were to detect *Klotho* gene polymorphisms (-395G>A SNP) and to identify their possible relationships with clinical findings in patients with chronic obstructive pulmonary disease (COPD). **Methods:** In 167 patients with COPD -395G>A SNP of the *Klotho* gene was genotyped by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) coupled with sequencing. The possible relationship was explored of -395G>A SNP with clinical findings such as lung function parameters, staging according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD), and body mass index (BMI). **Results:** Of the 167patients with COPD, 99 (59.3%) presented the wild type -395G allele, 62 (37.1%) were heterozygotes (–395GA allele), and 6 (3.6%) presented the non-wild type–395A allele. In these COPD patients there was an association between *Klotho* genotypes and BMI (p=0.025). No association was found between *Klotho* gene polymorphism and disease severity, assessed by spirometry, arterial blood gases and GOLD stage. **Conclusion:** *Klotho* -395G>A polymorphisms are detected in patients with COPD and are associated with BMI, but not with various parameters of disease severity. This may suggest a possible metabolic pathway in the implication of *Klotho* deficient gene in the pathophysiology of emphysema in COPD patients. ***Pneumon 2010, 23(4):348-354.***

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is the fourth leading cause of death in the USA[1], and leads to about one million deaths every year worldwide[2]. COPD is characterized by slowly progressive, at best partially

reversible airflow limitation, which is usually progressive and is associated with an abnormal inflammatory response[3,4]. COPD is strongly associated with smoking, since approximately 95% of patients have a history of tobacco use. Not all smokers, however, will develop COPD; it has been estimated that approximately 15-25% of smokers will develop this condition during their life. Although important progress has been made over the past few years, the pathophysiology of the disease is still unclear and is the subject of intensive research, especially with regard to the genetic determinants likely to predispose to disease development[5].

Klotho was identified by Kuro-o and associates[6] in a mouse model of aging and was named after the Greek goddess of fate (daughter of Zeus and Themis), who presided over birth and drew the thread of life from her distaff. Human *Klotho* shows 86% amino acid identity with the mouse protein, and is encoded by a gene that spans over 50 kb on chromosome 13q12[7]. The *Klotho* gene encodes a single-pass transmembrane protein, β-glycosidase enzyme, and functions as an aging-suppressor gene that extends life span when overexpressed and accelerates the development of aging-like phenotypes when disrupted[6,8]. A -395G>A promoter polymorphism in the *Klotho* gene has been associated with age related risk for development of several clinical conditions, including hypoactivity, muscle atrophy, skin atrophy, osteopenia, vascular calcification, soft tissue calcification, and pulmonary emphysema[6,8]. An electrophoretic mobility shift analysis revealed that the G–A substitution in the promoter region affects DNA – protein interaction. Mice homozygous for the mutation are defective in *Klotho* gene expression and exhibit a syndrome resembling human aging[9].

The mice homozygous (-395AA) for the *Klotho* transmutation ($KL^{-/-}$) showed short life expectancy and the beginnings of pulmonary emphysema that resembles human pulmonary emphysema both histologically and functionally[6]. The first histological changes appeared at 4 weeks of age, consisting of enlargement of the air spaces accompanied by destruction of the alveolar walls, and they progressed gradually with age[6,10]. The emphysematous changes were not caused by a developmental defect or hypoplasia of the lung. The lungs of the longer surviving mice (more than 120 weeks), heterozygous for the transmutation KL+/-, also developed emphysematous changes and alveolar calcification, almost identical to those found at an earlier stage in homozygous *Klotho* mice, suggesting a gene-dose effect of the *Klotho* gene on the development of lung lesions[10].

Based on these experimental studies, we hypothesised that genetic variations likely to regulate the levels of expression of the *Klotho* gene might be present in patients with COPD. The aims of this study were, therefore, to identify the distribution of -395G>A variant alleles in patients with COPD and to explore their possible relationship with clinical parameters such as disease severity, lung function, age, and BMI.

MATERIALS AND METHODS

Patients

Patients included in the study fulfilled the following criteria: COPD confirmed by medical history and by lung function tests (obstructive syndrome in the flow-volume curve), smoking at least 25 pack-years, Greek origin, and genetically unrelated to another study patient. Exclusion criteria were: diagnosis of pulmonary disease other than COPD, diagnosis of any collagen-related disease, diagnosis of malignancy, metabolic disturbances, and non Greek origin. Detailed medical history and tobacco exposure were recorded for each screened patient, who then underwent lung function testing including forced expiratory volume in the 1st second (FEV_1), forced vital capacity (FVC), FEV_1/FVC ratio and measurement of blood gases (PaO_2, $PaCO_2$, pH). The patients were classified for disease severity according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) staging[11]. Body mass index (BMI) was calculated for all patients by dividing weight (in kg) over height^2 (in m^2)[12]. From each patient who was enrolled in the study a sample of 10ml of peripheral blood was drawn into an EDTA tube for extraction of DNA from white cells.

The study was approved by the Ethics Committee of the University Hospital of Alexandroupolis and of the Medical School of the Democritus University of Thrace and written informed consent was obtained from each participant.

Of 235 patients with diagnosis of COPD who were screened during a one-year period, 167 patients fulfilled the study criteria and consented to participate: 162 males (97%) and 5 females (3%). The patients' characteristics are shown in Table 1.

Genetic analysis

DNA was extracted from peripheral blood white cells according to the standard salt extraction procedure.

Polymerase chain reaction-single strand conformation

TABLE1. The demographic characteristics of the study patients with chronic obstructive pulmonary disease (COPD)

	All patients with COPD (n=167)	
	Mean±SD	**Range**
Age (years)	69.4±8	46-85
Smoking (pack-years)	63.9±30.4	25-200
BMI (kg/m^2)	27.87±4.36	19.5-48.3
FEV1 % predicted	47.8±17	12-91
FVC % predicted	64±20	21-111
FEV1/FVC % predicted	47.9±17	12-91
PaO_2 (mm Hg)	66.4±10.3	44-99
$PaCO_2$(mmHg)	41.7±6.3	30-73
pH	7.41±0.3	7.31-7.52

BMI = body mass index, FEV_1 = forced expiratory volume in the 1st second, FVC = forced vital capacity

FIGURE 1. The three distinct single strand conformation polymorphism (SSCP) patterns of -395G>A genotypes.

polymorphism (PCR-SSCP) was used as the screening method for polymorphism -395G>A genotypes, coupled with sequence analysis using the forward primer 5′-TAGGGCCCGGCAGGAT-3′ and the reverse primer 5′-CCTGGAGCGGCTTCGTC-3′. Specifically, 5 microlitres of the PCR product were mixed with 5 μl of denaturing solution containing 95% deionized formamide, 0.05% bromophenol blue, 0.05% xylene cyanol and 20mM EDTA. The mixture was heated at 95°C for 5 min, then chilled on ice, and subsequently loaded on 12% nondenaturing polyacrylamide gel containing 5% glycerol. The gels were allowed to run at room temperature for 10 hours at 7V/cm. Bands were detected by silver staining. The SSCP pattern (Figure 1) corresponding to each -395G>A genotype was determined after sequencing of the PCR product by ABI 3700 DNA Automated Sequencer (Figure 2).

Statistical analysis

Statistical analysis was performed using StatView™ 4.5 software (Abacus Concepts Inc., Berkeley Ca.). Mean values were compared for data between the different study groups, with expression of standard deviation (±SD). The unpaired *t*-test was used to reveal potential significant differences in the distribution of -395G>A genotypes among the COPD patients. The analysis of variances (ANOVA) factorial test was employed to investigate possible associations between the clinical data (age, smoking, BMI, FEV_1, FVC, FEV_1/FVC, PO2, PCO2, pH and the three genotypes of -395G>A, using mean values and SD. The chi square test was used for comparison of genotype distributions among the parameters studied. For all tests statistical significance was set at $p \leq 0.05$.

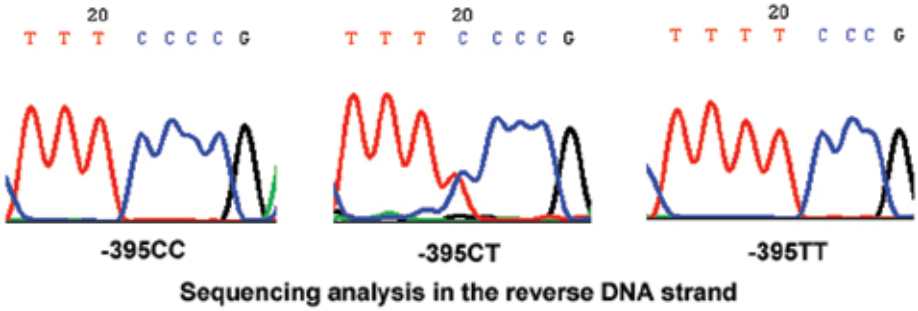

FIGURE 2. Sequencing analysis, revealing the three *Klotho* gene –395G>A genotypes: the sequencing analysis was better displayed in the reverse DNA strand revealing the three –395G>A genotypes as –395CC, -395CT, and -395TT.

RESULTS

Of the 167 study patients with COPD, 99 (59.3%) were homozygous for the wild type -395GG allele, 62 (37.1%) were heterozygotes (–395GA allele), and 6 (3.6%) carried in homozygosity the non-wild type–395AA allele.

The patients were correctly staged by the GOLD classification, since a significant association was found between the GOLD stage and the disease severity measured as a decline in FEV1 ($p<0.0001$). No relationship was observed between the GOLD classification and the distribution of -395G>A genotypes (chi square = 5.5, p=0.23). In addition, the -395G>A genotypes did not appear to influence the disease severity as assessed by the mean values of FEV1 (ANOVA factorial overall p=0.41), FVC (ANOVA factorial overall p=0.36), FEV1/FVC (ANOVA factorial overall p=0.44), PaO2 (ANOVA factorial overall p=0.27), PaCO2 (ANOVA factorial overall p=0.99) and pH (ANOVA factorial overall p=0.41) (Table 2).

A statistically significant distribution was observed of allelic genotypes in relation to BMI (ANOVA factorial overall p=0.025, Table 2) (Figure 3), specifically: patients homozygous for the non-wild-type (-395A allele) had a higher mean BMI (32.2±3.6 Kg/m^2) than both the homozygotes for the wild type (-395G allele) (28±4.6 Kg/m^2) (p=0.01) and the heterozygotes (-395GA allele) (27.2±3.7 Kg/m^2) (p=0.007). The distribution of the -395G>A genotypes was not related to age, (ANOVA factorial overall p = 0.55), or to the severity of smoking (ANOVA factorial overall p=0.11) in COPD patients.

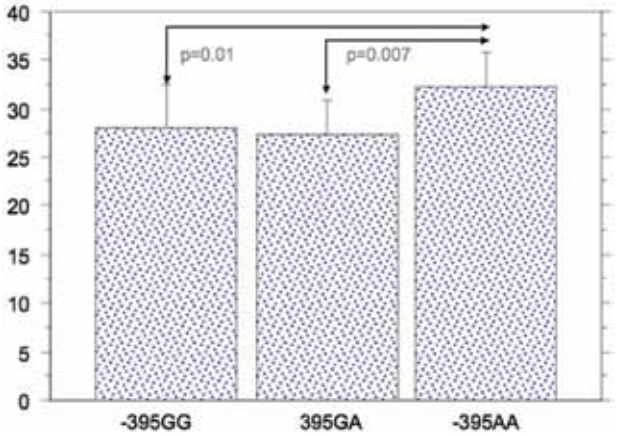

FIGURE 3. The mean body mass index (BMI) (kg/m^2) in the three *Klotho* gene -395G>A genotypic subgroups of patients (overall p = 0.025). Error bars show standard deviation.

DISCUSSION

This study simply provides the *Klotho* genotype frequencies in patients with COPD patients and explores possible associations with age, lung function, GOLD stages and BMI. No association with age, lung function or GOLD stages was found, but an association with BMI was observed. This is the first study reporting results of *Klotho* gene identification in a large group of patients with COPD. A low rate (6 patients - 3.6%) was found of homozygosity for the non-wild type–395AA allele, which is the deficient allele. The number of patients with heterozygosity for the allele -395GA phenotype was 62/167 (39.1%).

In mice, the heterozygote allele -395GA phenotype may also present emphysematous changes, suggesting a gene-dose effect of the *Klotho* gene on the development of lung lesions[10]. According to the Suga et al, pulmonary emphysema may be associated with an overexpression of mRNA surfactant protein A and type IV collagen[10]. How-

TABLE 2. Parameters studied in 167 patients with COPD according to *Klotho* gene –395G>A genotypes

Parameters	-395GG (n=99)	-395GA (n=62)	-395AA (n=6)	P
Mean age±SD (years)	69±8	70.1±8	67.3±10.3	0.55
Mean pack years±SD	68±33.2	57.9±25.3	58.5±21.4	0.11
Mean BMI±SD (kg/m^2)	28±4.6	27.2±3.7	32.2±3.6	0.025
Mean FEV1±SD (%pred)	48±16.5	46.7±17.8	56.3±15.6	0.41
Mean FVC±SD (%pred)	63.8±20.1	63.4±21.3	75.5±15.5	0.36
Mean FEV1/FVC±SD (%)	48±16.5	47±18	56.3±15.6	0.44
Mean PaO_2±SD (mmHg)	66±10	66.5±9.8	72.8±13	0.27
Mean $PaCO_2$±SD (mmHg)	41.7±6.3	41.6±6.4	41.8±6.2	0.99
Mean pH±SD	7.41±0.31	7.41±0.39	7.40±0.29	0.1

BMI = body mass index, FEV_1 = forced expiratory volume in the 1st second, FVC = forced vital capacity

ever, this activity may be a compensatory response to the destructive changes of the lung, and the pathogenesis of pulmonary emphysema in *Klotho* mice is still unclear. The imbalance of proteases and antiproteases is associated with the pathogenesis of pulmonary emphysema[4,5]. This mechanism was evaluated in animals, with the expression of matrix metalloproteinases 2 and 9 (MMP-2, MMP-9) and tissue inhibitor metalloproteinases 1 and 2 (TIMP-1, TIMP-2) in the lungs of *Klotho* mice[13], and confirmed in series of Japanese COPD patients with emphysema[14,15].

In the present study, no association of *Klotho* polymorphisms with demographic parameters such as age or gender was observed, although other studies showed an association of the *Klotho* gene with advanced age[16] or female gender[17,18]. Arking and associates[19] studied variations of the human *Klotho* locus, in order to establish a possible relationship with survival in Bohemian Czechs, Baltimore Caucasians and Afro-Americans, using microsatellite markers. They demonstrated significant differences in selected marker allele frequencies between newborn infants and elderly individuals that lead to the identification of an allele, the KL-VS, which influences the trafficking and catalytic activity of *Klotho* and concluded that variation in *Klotho* function contributes to heterogeneity in the onset and severity of human age-related phenotypes[19]. A possible explanation of the negative findings of the present study is that the study patients with COPD were all in a small age range (Table 1) and only 5 were female (3%), due to the low incidence of female smokers in Greece[20].

An association was demonstrated of the non-wild type allele –395A with increased BMI. A similar association has been reported in patients with coronary artery disease[21], but also in Japanese healthy males[22]. This novel finding suggests an important role of the *Klotho* gene in lipid metabolism[22]. *In vivo Klotho* gene delivery has been shown to ameliorate vascular endothelial dysfunction, increase nitric oxide (NO) production and prevent medial hypertrophy and perivascular fibrosis in a rat model with multiple atherogenic risk factors, including hypertension, diabetes mellitus, obesity, and hyperlipidaemia[23,24]. Recent experiments have shown that endothelium-dependent vasodilation of the arteries was impaired in heterozygous *Klotho* mice, but could be restored by parabiosis with wild-type mice[25]. In addition, NO metabolites (NO2 and NO3) in urine are significantly increased in non-wild-type *Klotho* mice[25]. Oxidative stress also plays a crucial role in the aging-associated cognition impairment process in *Klotho* mutant mice[26]. These results suggest that *Klotho* protein may protect through a process of endothelium-derived NO production[25]. Oxidative stress occurs when reactive oxygen species are produced in excess and lead to harmful effects, including damage to lipids, proteins and DNA. There is increasing evidence that oxidative stress is an important feature in COPD[4,5]. Several studies have demonstrated an increase of oxidants in the exhaled air[27] and urine[28] of patients with COPD. This could be another possible mechanism of development of emphysema associated with *Klotho* deficiency.

No vaiarion in disease severity was observed with respect to the -5G>A genotype distribution. None of the parameters of disease severity studied, such as the GOLD stage, FEV_1, FEV_1/FVC, or blood gases, showed any significant relationship with the *Klotho* -395G>A genotype distribution. A possible explanation for the absence of association is that the large majority of patients in this study had advanced stage disease, as shown in Figure 3, and had very similar lung function parameters. The cohort study in animals of Sato and associates showed that when emphysema appeared in *Klotho* mice, their total lung capacity (TLC) was significantly decreased compared to that of control mice[29]. Apart from the present study, there have been no reports on assessment of *Klotho* gene polymorphisms and lung function parameters in patients with COPD. In this study, no association was found between the *Klotho* -395G>A genotype distribution and smoking. In the Imamura study, smoking was shown to be an independent risk factor for coronary heart disease, in association with the -395A allele[21]. A possible association of the -395A allele with smoking in the occurrence of emphysema is under investigation in *Klotho* mice[10], and to date the only relevant data concerning patients with COPD are those reported in the present study.

Among the limitations of this study are the lack of emphysema comfirmation by HRCT in the study patients, and the absence of a control group. For selection of a control group it would be necessary to choose a great number of subjects matched for age, but without a history of smoking and suffering from no other disease related to *Klotho,* such as cardiovascular, metabolic, or osseous conditions[21,30,22]. It would be almost impossible to find enough subjects with those characteristics in this region. However, this study was not designed as a cohort study to compare with a control group. It simply aimed to depict *Klotho* alleles in the population of patients with COPD. The absence of emphysema confirmation by HRCT in the study patients was primarily a question of significant increase of the cost of the study, which would

be unacceptable by the Internal Review Board. Indeed, HRCT is helpful for scoring the severity of emphysema[31], but it is difficult to distinguish patients with emphysema from those with chronic bronchitis or severe asthma who also have a degree of emphysema[32]. Thus, HRCT is not indicated as a routine examination for diagnosis of COPD[11], although future studies are expected to establish its utility in routine clinical practice[32,33].

The mechanism of action of the *Klotho* gene is still under investigation. The data documenting the implication of the *Klotho* gene in the pathophysiology of emphysema to date has concerned only animal models. No previous study has investigated a possible association between the *Klotho* gene and the characteristics of patients with COPD. In the present study *Klotho* -395G>A polymorphism was detected in patients with COPD. With the exception of BMI, no other association was found with clinical parameters, specifically those assessing the severity of COPD, including lung function tests, GOLD staging, the age of the patient and the smoking history. Considering that *Klotho* gene is a metabolic gene, the question is raised as to whether the mechanism of emphysema induction of the *Klotho* deficient gene in patients with COPD is through a possible metabolic pathway.

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