



# Angiotensinogen gene G-6A polymorphism influences idiopathic pulmonary fibrosis disease progression

M. Molina-Molina<sup>\*,#</sup>, A. Xaubet<sup>\*,#</sup>, X. Li<sup>¶</sup>, A. Abdul-Hafez<sup>¶</sup>, K. Friderici<sup>+</sup>, K. Jernigan<sup>+</sup>, W. Fu<sup>§</sup>, Q. Ding<sup>§</sup>, J. Pereda<sup>\*,#</sup>, A. Serrano-Mollar<sup>#,f</sup>, A. Casanova<sup>\*\*</sup>, E. Rodríguez-Becerra<sup>##</sup>, F. Morell<sup>#,¶</sup>, J. Ancochea<sup>\*\*</sup>, C. Picado<sup>\*,#</sup> and B.D. Uhal<sup>¶</sup>

**ABSTRACT:** Angiotensin II is a growth factor that plays a key role in the physiopathology of idiopathic pulmonary fibrosis (IPF). A nucleotide substitution of an adenine instead of a guanine (G-6A) in the proximal promoter region of angiotensinogen (AGT), the precursor of angiotensin II, has been associated with an increased gene transcription rate.

In order to investigate whether the G-6A polymorphism of the AGT gene is associated with IPF development, severity and progression, the present study utilised a case–control study design and genotyped G-6A in 219 patients with IPF and 224 control subjects.

The distribution of G-6A genotypes and alleles did not significantly differ between cases and controls. The G-6A polymorphism of the AGT gene was not associated with disease severity at diagnosis. The presence of the A allele was strongly associated with increased alveolar arterial oxygen tension difference during follow-up, after controlling for the confounding factors. Higher alveolar arterial oxygen tension changes over time were observed in patients with the AA genotype ( $0.37 \pm 0.7$  mmHg ( $0.049 \pm 0.093$  kPa) per month) compared to GA genotype ( $0.12 \pm 1$  mmHg ( $0.016 \pm 0.133$  kPa) per month) and GG genotype ( $0.2 \pm 0.6$  mmHg ( $0.027 \pm 0.080$  kPa) per month).

G-6A polymorphism of the angiotensinogen gene is associated with idiopathic pulmonary fibrosis progression but not with disease predisposition. This polymorphism could have a predictive significance in idiopathic pulmonary fibrosis patients.

**KEYWORDS:** Angiotensin system, genetics, interstitial lung disease

Idiopathic pulmonary fibrosis (IPF) is a specific form of chronic interstitial lung disease, characterised histopathologically by usual interstitial pneumonia and associated with a poor prognosis [1]. IPF involves the loss of lung architecture through increased epithelial cell apoptosis and abnormal wound healing, followed by fibroblast foci formation and excessive lung collagen deposition. The triggering event is thought to be multiple microscopic sites of alveolar-capillary barrier injury, in which the disruption of microvascular endothelium and the apoptosis and activation of alveolar epithelium induce the secretion of profibrotic molecules, which in turn lead to the continuous fibrotic response and the progression of the disease [2, 3].

The initial cause of IPF remains unknown; however, it probably involves an interaction between environmental and genetic factors. Although the basis of individual susceptibility to lung fibrosis is unclear, familial clustering of

IPF has been described, even in individuals raised in different environments [4, 5]. Similar genetic mutations in affected family members have also been observed [6, 7], as well as in gene expression profiling of sporadic IPF [8, 9]. These observations, together with the failure of fibrogenic agents, such as asbestos, amiodarone or bleomycin, to trigger lung fibrosis in all exposed individuals [1] suggest that genetic factors play an important role in the development of pulmonary fibrosis. Among the large number of genes that could be involved in IPF susceptibility or progression, the most attractive targets may include the genes of mediators that influence the process of alveolar epithelial cell activation and lung wound healing [8–10].

Angiotensin (ANG) II is an essential mediator in the pathogenesis of pulmonary fibrosis. It has been shown that ANG II induces alveolar epithelial cell apoptosis, enhances fibroblast proliferation and lung collagen production, and

## AFFILIATIONS

<sup>\*</sup>Servicio de Neumología, Hospital Clínico, Instituto de Investigaciones Biomédicas Agustí Pi Suñer (IDIBAPS),

<sup>†</sup>Dept de Patología Experimental, Instituto de Investigaciones Biomédicas de Barcelona, Consejo Superior de Investigaciones Científicas,

<sup>\*\*</sup>Servicio de Neumología, Hospital Vall d'Hebron, Barcelona,

<sup>\*\*\*</sup>Servicio de Neumología, Hospital La Princesa, Madrid,

<sup>##</sup>Servicio de Neumología, Hospital Virgen del Rocío, Sevilla, and

<sup>¶</sup>Centro Investigaciones Biomédicas en Red (CIBER) de Enfermedades Respiratorias, Spain.

Depts of <sup>¶</sup>Physiology,

<sup>+</sup>Microbiology and Molecular Genetics, and

<sup>§</sup>Epidemiology, Michigan State University, East Lansing, MI, USA.

## CORRESPONDENCE

M. Molina-Molina, Servicio de Neumología, Hospital Clínico, c/Villarroel 170.08036, Barcelona, Spain.  
Fax: 34 2275455

E-mail: mariamolinalolina@hotmail.com

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## STATEMENT OF INTEREST

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increases transforming growth factor (TGF)- $\beta_1$  synthesis [11–13]. Angiotensinogen (AGT) is the precursor of ANG II. There is a significant increase of AGT expression in the lungs of IPF patients compared with normal lungs [14]. Furthermore, AGT has been found to be one of the most overexpressed genes in IPF lungs compared to the gene expression profile of other interstitial lung diseases [8]. A modest increase in basal expression of AGT may lead to a chronic rise in baseline ANG II production. Recently, it has been observed that the inhibition of AGT mRNA expression attenuates bleomycin-induced lung fibrosis [15]. The regulation of cytokine production in individuals has a major genetic component. The human gene encoding AGT is located on chromosome 1q42.3 and a large number of single-nucleotide polymorphisms (SNPs) have been described. Among them, the SNP G-6A nucleotide substitution at position -6 upstream from the initial transcription start has been studied particularly. The A allele has been associated *in vitro* with an increased expression of the AGT gene and with higher AGT synthesis [16]. Given the proven relationship between AGT and IPF, the present authors hypothesised that changes in the expression of AGT gene may be involved in the pathogenesis of the disease. Accordingly, the current study investigated whether the SNP G-6A is associated with the development and progression of IPF.

## MATERIALS AND METHODS

### Subjects

The control group was composed of 224 unrelated healthy subjects (mean  $\pm$  SD age  $40 \pm 13$  yrs; 80 males and 144 females) with no associated medical disease. White Caucasian subjects from Spain were selected for the study in four different hospitals (Hospital Clínico and Hospital Vall d'Hebron, Barcelona; Hospital La Princesa, Madrid; and Hospital Virgen del Rocio, Sevilla, Spain). Any subjects with diseases related to potential tissue fibrosis were excluded from the study. The representative nature of the control group for the Spanish population has been previously demonstrated in human leukocyte antigen genotyping studies [17]. The IPF group was composed of 219 unrelated patients (age  $67.3 \pm 10$  yrs; 136 males and 83 females). The diagnosis of IPF was established according to the American Thoracic Society/European Respiratory Society Consensus Statement [1]. Histological diagnosis was obtained in 89 (40.6%) out of the 219 patients. Pulmonary function tests were performed as previously described and the reference values used were from the present authors' laboratory [18]. The alveolar–arterial oxygen tension difference ( $PA-aO_2$ ) was calculated according to the standard formula, using the actual respiratory exchange ratio [19]. Among the IPF patients, 181 were followed up for  $33 \pm 23$  months; the following included the assessment of clinical data and lung function. The main reason for subjects discontinuing with the study was death. The Human Ethics Committee at each participating hospital approved the study and written informed consent was obtained from all subjects.

### Identification of the polymorphism and primer sequences

Genomic DNA was extracted from peripheral leukocytes by a salting out procedure [20]. PCR was used to assay G-6A alleles and genotypes of each studied subject, using the following primers: 5'-forward-GTC GCT TCT GGC ATC TGT CC-3'; 5'-reverse-CTT TTT CCT CCT AGC CCA CA-3'. PCR cycling

conditions were as follows: 94°C for 5 min, followed by 35 cycles, each at 90°C for 30 s, 65°C for 30 s, and 72°C for 45 s, and a final extension at 72°C for 7 min. The product was digested with BstNI (NEB, Beverly, MA, USA) at 60°C for 2–3 h, followed by electrophoresis on a 3% agarose gel, yielding the following products: -6G: 173, 86 bp and -6A: 119, 86 and 55 bp.

### Statistical analysis

Hardy–Weinberg Equilibrium held for each genotype within the control group using the Chi-squared test with the appropriate number of degrees of freedom [21]. Differences in genotype and allele frequencies between case and control groups were analysed with the Pearson Chi-squared test and data were corrected by multiple comparisons. A logistic regression model was fitted to the IPF data with SNP genotype or allele effect of G-6A, with age and sex as covariates. Differences in the mean values of continuous variables were tested with an unpaired t-test or the Wilcoxon signed rank test. The relationships between the progression of IPF (changes in pulmonary function tests over time), the treatment, and the type of genotype and allele were tested by ANCOVA, adjusting the initial measure of the pulmonary function at diagnosis. Results of disease progression were adjusted by age, sex and smoking variables. Gauss–Markov conditions on model residuals were examined and outliers were excluded to ensure the model fitted. Data are expressed as mean  $\pm$  SD. A p-value of  $<0.05$  was considered statistically significant.

## RESULTS

### G-6A gene polymorphism in IPF patients and control subjects

The genotype and allele frequencies of G-6A polymorphism of AGT gene are summarised in table 1. There were no significant differences in genotype and allele frequencies between the control subjects and patients with IPF (genotypes: Chi-squared=1.25,  $p=0.53$ ; alleles: Chi-squared=0.82,  $p=0.36$ ). Both age and sex had significant association with a predisposition for IPF; the risk of developing the disease was higher in males ( $p<0.0087$ ) and older patients ( $p<0.0001$ ). Neither SNP genotype nor allele of the -6ATG gene polymorphism was significant in the development of IPF after adjusting for patient age and sex. The reported genotype and allelic frequencies of

**TABLE 1** G-6A genotype and allele frequencies

|                   | Control subjects | IPF patients   |
|-------------------|------------------|----------------|
| <b>Subjects n</b> | 224              | 219            |
| <b>Genotypes</b>  |                  |                |
| AA                | 19.2 (43/224)    | 18.2 (40/219)  |
| GA                | 53.6 (120/224)   | 52.5 (115/219) |
| GG                | 27.2 (61/224)    | 29.2 (64/219)  |
| <b>Allele</b>     |                  |                |
| A                 | 46 (206/448)     | 47 (207/438)   |
| G                 | 54 (242/448)     | 53 (231/438)   |

Data are presented as % (n/total n), unless otherwise stated. IPF: idiopathic pulmonary fibrosis.

G-6A gene polymorphisms in the control group did not differ from those previously described in white Caucasian populations [22]. No deviations from Hardy–Weinberg Equilibrium were seen in either male or female control groups. There was no evidence for any interaction between sex and allele frequencies ( $p=0.48$ ). There were no differences in allele distribution across age groups in control and IPF populations. Therefore, the G-6A polymorphism was not associated with susceptibility to develop IPF.

#### IPF severity and disease progression related to genotype and allele frequencies of G-6A gene polymorphism

The respiratory functional characteristics of the IPF patients are summarised in table 2. Baseline pulmonary functional test results did not significantly differ between G and A alleles. Likewise, there was no significant correlation among G-6A genotypes and baseline lung functional test values.

In total, 181 patients were monitored for  $33 \pm 23$  months (range 3–114 months). Contact with the remaining 38 patients was lost. Overall, patients showed deterioration in pulmonary functional parameters (table 3). Changes in pulmonary function tests during the follow-up were expressed over time to compare evolution between patients. The changes in pulmonary function tests were divided by the whole follow-up period in months. Decline in pulmonary functional values was not significantly different between males and females (table 3). A total of 130 monitored patients were treated with glucocorticoids, with or without azathioprine or cyclophosphamide and N-acetylcysteine. The remaining 51 patients did not receive medical treatment during the monitoring period. There were no differences in the changes in pulmonary functional test results between treated and untreated patients during the evaluated months (table 4).

There was a strong association between the presence of the A allele and the monthly increase in  $PA_{-a,O_2}$  ( $0.83 \pm 0.92$  mmHg ( $0.11 \pm 0.12$  kPa) versus  $0.53 \pm 0.7$  mmHg ( $0.070 \pm 0.093$  kPa);  $p=0.001$ ). Patients with the A allele who did not receive treatment during follow-up presented higher  $PA_{-a,O_2}$  than

patients with the A allele who were treated ( $p=0.01$ ). Significant differences were observed in the  $PA_{-a,O_2}$  changes during the follow-up between patients with the AA genotype of the AGT gene ( $0.36 \pm 0.7$  mmHg ( $0.048 \pm 0.093$  kPa) per month) and those with GA genotype ( $0.12 \pm 1$  mmHg ( $0.016 \pm 0.13$  kPa) per month) as well as in those with GG genotype ( $0.2 \pm 0.6$  mmHg ( $0.027 \pm 0.08$  kPa) per month;  $p=0.0015$  and  $p=0.005$ , respectively; table 5). There were no differences between the follow-up duration in patients with A and G alleles ( $35.8 \pm 27.7$  months versus  $31.7 \pm 19.8$  months, respectively). The carriage of the G allele in IPF patients was associated with less deterioration of forced vital capacity (FVC) but the difference was not significant ( $p=0.09$ ). The decrease in FVC over time was not significantly different between G-6A genotypes (table 5). No differences in the diffusion capacity of the lung for carbon monoxide ( $DL_{CO}$ ) and  $DL_{CO}$  adjusted by alveolar volume (KCO) were found between genotypes during follow-up (table 5). However, a greater change in KCO was observed over time in male patients carrying an A allele ( $p=0.05$ ).

#### DISCUSSION

The present study demonstrates, for the first time, the relationship between the G-6A polymorphism of the AGT gene and IPF disease progression. The results show that the G-6A gene polymorphism does not confer susceptibility to IPF, as no differences are seen in the distribution of genotypes and allele frequencies between patients and healthy subjects. However, the presence of the A allele and AA genotype at the -6 position in the core promoter of the AGT gene is associated with an increased deterioration in gas exchange and pulmonary function in patients with IPF. This finding suggests a potential use for the G-6A gene polymorphism as a prognostic marker in IPF to predict poor outcome and to aid selection of the appropriate therapeutic intervention.

Several studies have been undertaken in IPF patients in order to find genetic links to shed more light on the disease's pathogenesis and suggest target pathways for therapy [10, 23]. The pathophysiology of IPF is likely to be determined by multiple genetic factors that each contribute to disease development [10, 23]. Following the evolving hypothesis, which suggests that IPF is a consequence of impaired wound

**TABLE 2** Baseline pulmonary function test findings in patients with idiopathic pulmonary fibrosis

|   | Absolute         | % pred            |
|---|------------------|-------------------|
| FVC L   | $2.37 \pm 0.7$   | $71.66 \pm 17.24$ |
| FEV <sub>1</sub> L  | $1.95 \pm 0.5$   | $78.51 \pm 18.13$ |
| FEV <sub>1</sub> /FVC % pred                                  | $83.5 \pm 0.05$  |                   |
| TLC L   | $3.94 \pm 1.1$   | $71.91 \pm 15$    |
| $DL_{CO}$ mL·min <sup>-1</sup> ·mmHg <sup>-1</sup>            | $13.37 \pm 0.51$ | $57.89 \pm 1.78$  |
| Kco mL·min <sup>-1</sup> ·mmHg <sup>-1</sup> ·L <sup>-1</sup> | $3.73 \pm 0.1$   | $79.86 \pm 2.36$  |
| $Pa_{a,O_2}$ mmHg   | $75.09 \pm 0.14$ |                   |
| $PA_{-a,O_2}$ mmHg  | $27.22 \pm 1.12$ |                   |

Data are presented as mean  $\pm$  SD. % pred: % predicted; FVC: forced vital capacity; FEV<sub>1</sub>: forced expiratory volume in one second; TLC: total lung capacity;  $DL_{CO}$ : diffusion capacity of the lung for carbon monoxide; Kco: ratio between  $DL_{CO}$  and alveolar volume;  $Pa_{a,O_2}$ : arterial oxygen tension;  $PA_{-a,O_2}$ : alveolar–arterial oxygen tension difference. 1 mmHg=0.133 kPa.

**TABLE 3** Changes in pulmonary function tests in the follow-up study

|                    | Values          | Males           | Females          |
|--------------------|-----------------|-----------------|------------------|
| Patients n         | 181             | 107             | 74               |
| FVC                | $-0.47 \pm 1.2$ | $-0.49 \pm 1.3$ | $-0.45 \pm 0.87$ |
| $DL_{CO}$          | $-0.72 \pm 1.4$ | $-0.78 \pm 1.8$ | $-0.70 \pm 1.5$  |
| Kco                | $-0.2 \pm 0.9$  | $-0.27 \pm 0.8$ | $-0.18 \pm 1.1$  |
| $PA_{-a,O_2}$ mmHg | $0.19 \pm 0.9$  | $0.32 \pm 0.8$  | $0.12 \pm 0.6$   |

Unless otherwise stated, data are presented as the mean  $\pm$  SD percentage of change in absolute values or difference in pressure (mmHg) with respect to initial assessment, divided by the length of follow-up in months. FVC: forced vital capacity;  $DL_{CO}$ : diffusion capacity of the lung for carbon monoxide; Kco: ratio between  $DL_{CO}$  and alveolar volume;  $PA_{-a,O_2}$ : alveolar arterial oxygen tension difference. 1 mmHg=0.133 kPa.

**TABLE 4** Changes in pulmonary function test results in treated and untreated patients with idiopathic pulmonary fibrosis over time

|   | Treated     | Untreated   | p-value |
|---|-------------|-------------|---------|
| <b>Patients n</b>                         | 130         | 51          |         |
| <b>FVC</b>                                | -0.52 ± 1.1 | -0.44 ± 1.1 | NS      |
| <b>DL<sub>CO</sub></b>                    | -0.78 ± 1.8 | -0.83 ± 1.8 | NS      |
| <b>KCO</b>                                | -0.04 ± 1.9 | -0.18 ± 1.8 | NS      |
| <b>PA-a<sub>2</sub>O<sub>2</sub> mmHg</b> | 0.22 ± 0.95 | 0.2 ± 0.89  | NS      |

Unless otherwise stated, data are presented as the mean ± SD percentage of change in absolute values or difference in pressure (mmHg) with respect to initial assessment, divided by the length of follow-up in months. FVC: forced vital capacity; NS: not significant; DL<sub>CO</sub>: diffusion capacity of the lung for carbon monoxide; KCO: ratio between DL<sub>CO</sub> and alveolar volume; PA-a<sub>2</sub>O<sub>2</sub>: alveolar arterial oxygen tension difference. 1 mmHg=0.133 kPa.

healing involving the epithelial/fibroblast pathway [2], many candidate genes for growth factors, as well as other molecular mediators implicated in this extensive process, have been evaluated [24–28]. The first candidate genes to be analysed in IPF patients were involved in inflammatory responses. Later studies focused on surfactant protein gene variants, T-helper cell type 1/type 2 cytokine polymorphisms and growth factors involved in the epithelial/fibroblast altered pathway. Tumour necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 receptor antagonist, surfactant A and B protein and complement receptor 1 gene polymorphisms have been associated with increased risk of developing pulmonary fibrosis [24–28]. PANTELIDIS *et al.* [27] have shown that there is a strong correlation between the cocarriage of IL-6 and TNF- $\alpha$  receptor II genetic variants and IPF [27]. The presence of TGF- $\beta$ <sub>1</sub> and IL-6 polymorphisms have been linked with IPF progression due to their association with lung function deterioration over time [25, 27].

ANG II is a growth factor that plays an important role in the fibrogenic process of different organs. It is a crucial factor in the pathogenesis of lung fibrosis due to its contribution to abnormal wound healing [13]. *In vitro* and *in vivo* studies have shown that ANG II induces epithelial cell apoptosis, increases lung collagen deposition and enhances TGF- $\beta$ <sub>1</sub> synthesis [11, 13], whereas its inhibition or antagonism attenuates experimental lung fibrogenesis [12]. It has been shown that alveolar epithelial cell apoptosis induced by Fas ligand, TNF- $\alpha$ , bleomycin or amiodarone requires ANG II generation, while a blockade of ANG II production or receptor interaction can block cell death [11–13]. ANG-converting enzyme (ACE), the main enzyme involved in ANG II synthesis, is found at higher levels in the bronchoalveolar lavage fluid of patients with interstitial lung diseases and higher levels of ACE have been observed in the fibrotic stage of sarcoidosis [29]. It has recently been shown that ANG II and AGT are overexpressed in lungs of IPF patients, with a strong linkage with alveolar apoptotic epithelial cells and myofibroblasts [14]. Interestingly, the treatment of IPF fibroblast cultures with the ANG II antagonists decreases TGF- $\beta$ <sub>1</sub> synthesis and lung collagen deposition [13].

**TABLE 5** Changes in pulmonary function tests between G-6A genotypes during follow-up study

|   | AA          | GA                    | GG                     |
|---|-------------|-----------------------|------------------------|
| <b>Patients n</b>                         | 32          | 87                    | 62                     |
| <b>FVC</b>                                | -0.73 ± 1   | -0.35 ± 1.3           | -0.5 ± 1               |
| <b>DL<sub>CO</sub></b>                    | -0.7 ± 0.8  | -0.8 ± 1.6            | -0.6 ± 1.5             |
| <b>KCO</b>                                | -0.22 ± 0.6 | -0.26 ± 1.2           | -0.15 ± 0.8            |
| <b>PA-a<sub>2</sub>O<sub>2</sub> mmHg</b> | 0.36 ± 0.7  | 0.12 ± 1 <sup>#</sup> | 0.2 ± 0.6 <sup>†</sup> |

Unless otherwise stated, data are presented as the mean ± SD percentage of change in absolute values or a difference in pressure (mmHg) with respect to initial assessment, divided by the follow-up in months. FVC: forced vital capacity; DL<sub>CO</sub>: diffusion capacity of the lung for carbon monoxide; KCO: ratio between DL<sub>CO</sub> and alveolar volume; PA-a<sub>2</sub>O<sub>2</sub>: alveolar arterial oxygen tension difference. 1 mmHg=0.133 kPa. <sup>#</sup>: p=0.0015; <sup>†</sup>: p=0.005.

The genetic variability of ANG II synthesis could lead to the identification of subjects with a higher risk of ANG II effects. The present authors investigated the G-6A polymorphism in the core promoter region of the AGT gene, which is known to be the main regulator of AGT expression and synthesis [16]. The G-6A polymorphism was found to influence disease progression in IPF patients. The presence of the A allele and AA genotype is strongly correlated with PA-a<sub>2</sub>O<sub>2</sub> gradient deterioration over time. It is well known that the rate of decline in pulmonary function across the IPF population is not linear, so the present findings are not able to evaluate acute exacerbations or periods with no change over time in these patients. Since A allele carriage and AA genotype have been associated with higher AGT levels, it may well be that increases in ANG peptides influence the evolution of the disease. Previous work in liver fibrosis has described a positive association between -6 AA gene variants and an advanced grade of fibrosis, although genotype frequencies were not different from those of the control population [22]. Studies of ANG system gene polymorphisms in lung diseases have revealed that there is an increase in the D allele of the ACE gene in pulmonary fibrosis and in noninfectious pulmonary dysfunction that leads to allogenic stem cell transplant [30, 31]. Therefore, these results, together with the present findings, suggest that ANG system gene variants could be involved in interstitial lung fibrotic diseases.

In conclusion, the results of the present study raise the possibility that the G-6A polymorphism plays an important role in gas exchange and lung function deterioration in patients with idiopathic pulmonary fibrosis. The present study is the first to link the genetic variations in angiotensinogen with disease progression. Knowledge of this polymorphism may have a predictive significance in patients with idiopathic pulmonary fibrosis, and physicians may be able to use more aggressive therapy in those patients with a high risk of disease progression.

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