HHS Public Access

Author manuscript

Lung. Author manuscript; available in PMC 2015 June 22.

Published in final edited form as:

Lung. 2013 August ; 191(4): 353–360. doi:10.1007/s00408-013-9476-2.

ANGIOTENSINOGEN PROMOTER POLYMORPHISMS PREDICT LOW DIFFUSING CAPACITY IN U.S. AND SPANISH IPF COHORTS

My-Trang T. Dang[*,1], **Chenyang Gu**[*,2], **Jeannie I. Klavanian**[*,3], **Katherine A. Jernigan**[*,1], **Karen H. Friderici**[*,1], **Yuehua Cui**[*,2], **Maria Molina-Molina**[4], **Julio Ancochea**[4], **Antoni Xaubet**[6], and **Bruce D. Uhal**[*,3]

[*]Michigan State University, East Lansing, Michigan

[1]Department of Microbiology and Molecular Genetics, Hospital Universitario de Bellvitge, IDIBELL, Hospitalet del Llobregat. Barcelona, Spain and Centro de Investigación Biomédica en Red de Enfermedades Respiratorias (CIBERES)

[2]Department of Statistics and Probability, Hospital Universitario de Bellvitge, IDIBELL, Hospitalet del Llobregat. Barcelona, Spain and Centro de Investigación Biomédica en Red de Enfermedades Respiratorias (CIBERES)

[3]Department of Physiology, Hospital Universitario de Bellvitge, IDIBELL, Hospitalet del Llobregat. Barcelona, Spain and Centro de Investigación Biomédica en Red de Enfermedades Respiratorias (CIBERES)

[4]Servicio de Neumología, Hospital Universitario de Bellvitge, IDIBELL, Hospitalet del Llobregat. Barcelona, Spain and Centro de Investigación Biomédica en Red de Enfermedades Respiratorias (CIBERES)

[5]Servicio de Neumología, Hospital la Princesa, Madrid, Spain

[6]Servicio de Neumología, Institut Clínic del Tórax, Hospital Clínic, Barcelona, Spain and Centro de Investigación Biomédica en Red de Enfermedades Respiratorias (CIBERES).

Abstract

Purpose—Single nucleotide polymorphisms in angiotensinogen at positions -20 and -6 are associated with increased severity and progression of various fibrotic diseases. Our earlier work demonstrated that the progression of Idiopathic Pulmonary Fibrosis was associated with the A-6 allele. This study examined the hypothesis that the homozygous CC genotype at -20 and the AA genotype at -6 would confer worse measures of pulmonary function (measured by pulmonary function tests) in Idiopathic Pulmonary Fibrosis.

Address all correspondence and reprint requests to: Bruce D. Uhal, Ph.D. Michigan State University, Department of Physiology 3197 Biomedical and Physical Sciences Building East Lansing, MI 48824 Phone: (517) 355 – 6475 x1144 Fax: (517) 355 – 5125 uhal@msu.edu.

Conflict of Interest Statement

All the authors declare they have no Conflict of Interest for this manuscript.

Method—Multiple logistic regression analysis was applied to a NIH Lung Tissue Research Consortium cohort and Spanish cohort, while also adjusting for covariates to determine the effects of these SNPs on measures of pulmonary function.

Results—Analysis demonstrated that the CC genotype at -20 was strongly associated with reduced diffusing capacity in males in both cohorts ($p = 0.0028$ for LTRC and $p = 0.017$). In females, the AA genotype was significantly associated with lower FVC ($p = 0.0082$) and V_{alv} ($p = 0.022$). In males, the haplotype CA at -20 and -6 in AGT was also strongly associated with reduced diffusing capacity in both cohorts.

Conclusions—This study is the first to demonstrate an association of angiotensinogen polymorphisms (-20A>C and -6G>A) with lower measures of pulmonary function in Idiopathic Pulmonary Fibrosis. It is also the first to relate the effect of gender in lung fibrosis with polymorphisms in angiotensinogen.

Keywords

angiotensin II; fibrosing alveolitis; interstitial lung disease

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is the most common form of interstitial lung disease. It is a “chronic, progressive, and irreversible” condition with a bias towards males and people in their fifth through eighth decade of life [1]. Upon diagnosis, the mean survival is three years. Currently, the only therapy to prolong survival is lung transplantation [1]. However, the five year post-operative survival rate is 44% [1] The current modes of therapy (corticosteroids and immunosuppressants) are of minimal benefit to IPF patients. This reflects the incomplete knowledge underlying the pathogenesis of IPF and paves way for novel therapies to address this void [2].

A strong predictor of mortality in IPF is the number of myofibroblastic foci [3]. Myofibroblasts play an important role in lung fibrosis. They can be derived from a variety of sources including pericytes, fibrocytes, epithelial or endothelial cells, and normal lung fibroblasts, which when stimulated with transforming growth factor (TGF)-β_1 differentiate into myofibroblasts. Myofibroblasts within many tissues are a known source of collagen and angiotensin II (ANGII), and the ANGII produced by myofibroblasts is known to mediate fibrogenesis in various organ systems such as the heart, kidney, liver, pancreas, skin, and lung [4-10]. ANGII is derived from its precursor angiotensinogen (AGT), and both AGT and ANGII have been shown by this laboratory to be required for experimental lung fibrosis [11-12]. ANGII also enhances TGF-β_1 synthesis in human lung myofibroblasts isolated from patients with IPF [9]. In turn, TGF-β_1 is able to stimulate AGT transcription in myofibroblasts, thus creating an “ANGII-TGF- 1 autocrine loop” in myofibroblasts. [9] This laboratory also demonstrated that TGF-β_1 inducible AGT transcription is regulated through two transcription factors, JunD and HIF-1α, both of which act on binding domains in the core promoter of AGT in the region spanning from –46 to +22 from the transcription start site [13].

The core promoter also contains three single nucleotide polymorphisms (SNPs) located at -20, -18, and -6. The SNPs at these locations have been shown to result in changes in AGT transcription rate in non-pulmonary cell types. In hepatocytes, the presence of the CC haplotype at -20 and -18 respectively, increased AGT transcription to more than two-fold when compared to the AT haplotype [14]. Similarly, the presence of the A allele at -6 increased AGT transcription in comparison to the G allele at the same locus [15]. These SNPs have also been associated with the severity and/or progression of various diseases including IgA nephropathy, hepatic fibrosis and cirrhosis, hypertension, and IPF [16-21]. In a Spanish IPF cohort, our lab demonstrated that the AA genotype of -6G>A was significantly associated with disease progression as measured by alveolar-arterial oxygen gradient over time [20].

On this basis, it was hypothesized here that the presence of CC genotype at -20 and/or the AA genotype at -6, particularly when found together, would confer lower measures of pulmonary function in IPF as measured by pulmonary function tests (PFTs). In accord with this hypothesis, it was theorized that the presence of both of these alleles would confer a “risk haplotype” for IPF; the risk haplotype was predicted to be CA (at the -20 and -6 loci, respectively).

METHODS

Subjects

The Lung Tissue Research Consortium (LTRC) provided 163 blood samples and over 1100 associated clinical variables from IPF patients. From these, samples that were unable to be genotyped and samples that were missing variables of interest were excluded. The final pool consisted of 149 samples and 68 variables of interest that came from the categories of demographics, tobacco use, environmental exposure, disease history, medications, pulmonary function tests, and arterial blood gases. This pool was composed of 94 males and 55 females (mean ± SD age: 63.4 ± 8.5 and 62.4 ± 9.2 respectively). Similar analyses were performed on a second cohort consisting of 203 patients from a Spanish population. This group was composed of 123 males and 80 females (mean ± SD age: 66.1 ± 10.6 and 67.5 ± 13.1 respectively).

Genotyping polymorphisms at -20 and -6

The genotyping protocol was derived from Jeunemaitre et al. with modifications in primer design [22]. The primers utilized were 5′- GTC GCT TCT GGC ATC TGT CC -3′ (forward) and 5′- CCT TTT CCT CCT AGC CCA CA -3′ (reverse). A salting procedure was used to extract genomic DNA from peripheral leukocytes (23). Each sample was subjected to the following PCR cycling conditions: 94°C for 5 minutes, followed by 35 cycles, each at 94°C for 30 seconds, 63°C for 30 seconds, and 72°C for 45 seconds, with a final extension at 72°C for 7 minutes. Each reaction was performed in a 20 μL volume containing 0.5U Taq polymerase (Promega Corp, Madison, WI), 1.5 mM $MgCl_2$, 0.2 mMdNTPs, 0.1 μg/μLPuregeneRNAse A Solution (Gentra Systems, Minneapolis, MN), and 1 μM of each primer. The amplification of each product was checked on a 2% agarose gel using 5 μL of the PCR product. If amplification was sufficient, the remaining 15 μL underwent a

purification step to remove contaminating primers and dNTPs. The purification step consisted of adding 0.45 μL (5 U/μL) of Antarctic Phosphatase (New England Biolabs), 1.5 μL of 10x Antarctic Phosphatase buffer (New England Biolabs), and 0.225 μL (10 U/μL) of Exonuclease I (USB). This mixture was incubated at 37°C for 30 minutes followed by a 20 minute incubation at 80°C. Sequencing was performed using 2 μL of purified PCR product, 9.7 μL of water, and 0.3 μL (100 μM) of primer. Both forward and reverse primers were utilized in separate reactions and sequenced on an ABI Prism 3700 DNA Analyzer at the Research Technology Support Facility at Michigan State University. The results were analyzed using the program, Sequencher v4.7(Gene Codes Corp., Ann Arbor, MI) to determine SNPs rs 5050 (-20G>A) and rs 5051 (-6A>C) located in the promoter of AGT.

Statistical Analysis

The relationship between measures of pulmonary function (as measured by PFTs including FEV_1, FVC, FEV_6, mean DLCO, V_{alv}, and KCO) and the genotyped SNPs at -20 and -6 was tested by fitting regression models assuming different gene action modes (i.e., additive, dominance and recessive) after adjusting for the effects of covariates. Data analysis was done with statistical software R (2.13.2 version). From the original 163 individuals, the pool of 149 individuals was included for analysis after exclusion of samples that were unable to be genotyped and those in which variables of interest were missing. To account for possible gender differences in disease progression, missing phenotypic values were imputed using the mean value of the corresponding phenotype for male and female data separately. Analysis for the Spanish cohort was similar except for the inclusion of covariates (due to the lack of collection of these variables).

All the phenotypes (as measured by PFTs) were individually analyzed. A stepwise variable selection was initially performed on all covariates for each phenotype in R. The selected covariates were then fitted into the genetic models (Table S1) together with the SNP variables. For each phenotype, roughly ten to fifteen covariates were left after variable selection. Three genetic models representing different gene action modes were considered in this study (the joint model analyzing the whole population in Table S1). In practice, the true disease model is unknown. Statistically, a model selection criterion can be used to choose which genetic model fits the data best. The Akaike information criterion (AIC) was chosen to select the optimal model, which is defined as $AIC = -2logL + 2k$, where L is the regression likelihood and k is the total number of parameters fitted in the model. The one with the minimum AIC value is chosen as the optimal one. For the three models, testing a SNP effect is equivalent to testing $H_0: \beta_1 = \beta_{12} = 0$, a 2 degrees of freedom (*df*) likelihood ratio test, while adjusting for the effects of other covariates. The likelihood ratio statistic asymptotically follows a chi-square distribution with 2 *df*. For each phenotype, multiple testing adjustment was done for the two loci. Thus any SNP with a p-value < 0.025 was considered statistically significant by maintaining a family-wise error rate of 0.05. To assess if male and female populations have different genetic bases in determining worse measures in pulmonary functions, the above models were modified by removing the sex covariate as well as the genetic-by-sex interaction term (the sex-specific model in Table S1) and were fitted to the male and female data separately. The same set of covariates fitted with the male and female combined data was fitted into the modified models. Likelihood ratio test was

applied to test the significance of the regression coefficients after selecting the optimal model by the AIC criterion. A power study revealed that the datasets have more than 90% power to detect a mean difference larger than 0.7 between the largest and smallest means among the three genotype groups with a sample size of 55, the smallest sample size (for female in the LTRC cohort).

RESULTS

Characteristics of the patient population

Table 1a and 1b summarizes the age and mean pulmonary function test values for the LTRC and Spanish population subjects for which all genotyping and function test data were available. The data are separated by gender in accord with the finding of gender-specific differences in the association between AGT genotype and PFT values to be discussed below. No statistically significant differences were observed between males and females in any of the data reported in Table 1.

Genotype and allele frequencies

The genotype and allele frequencies for the -20A>C and -6G>A polymorphisms in AGT are summarized in Tables 2 and 3. No statistically significant differences were observed in the allele frequencies at either the -20 or -6 loci between men and women (Table 3).

Influence of AGT genotype on pulmonary function tests- whole population analysis

In an analysis of each cohort s a whole (Table 4, i.e. without separation by gender), the CC genotype at -20 was most strongly associated with reduction of KCO in both cohorts. However, the impact of gender on this measure was also significant (see below). The AA genotype at -6 also associated with reduction of KCO. In the LTRC cohort, gender did not influence this measure. Table 4 lists only those PFT data for which statistically significant differences were observed in this analysis.

Influence of AGT genotype on pulmonary function tests- gender-specific analyses

When the whole population was reanalyzed with sample separation on the basis of gender, several gender-specific effects of AGT genotype on PFT values were revealed. In the male IPF population (Table 5a), the CC genotype at the -20 locus was associated with a very strong reduction in KCO (from 3.19 ± 0.84 to 1.46 ± 0.34) of high statistical significance ($p = 0.0028$) in the LTRC cohort. This effect was also seen in the Spanish cohort [Table 5b ($p = 0.017$)]. The AA genotype at -6 also associated with reduced KCO in males, but with lower statistical significance ($p=0.0214$) in the LTRC cohort.

In females (Table 6), significant associations were only seen at the -6 locus. The -20 locus had no apparent effect. The AA genotype at -6 was associated with a reduction in FVC ($p = 0.0081$) and V_{alv} ($p = 0.022$) in the LTRC cohort. However, in the Spanish cohort, this genotype was associated with an increase in diffusing capacity ($p = 0.023$).). As discussed further below, the lack of decrease in KCO may be related to the large decrease in V_{alv} associated with the AA genotype at -6 in females ($p = 0.022$).

Analysis of an "IPF risk haplotype"

Multiple loci analysis revealed that in males, the AGT haplotype CA (at -20 and -6, respectively) was strongly associated with reduced KCO in both the LTRC cohort ($p = 0.0048$) and the Spanish cohort ($p = 0.014$). This association was not statistically significant in females. Interestingly, the AG haplotype at -20 and -6 also was associated with reduced KCO in males, but at lower statistical significance ($p=0.031$) in the LTRC cohort. When the male and female combined data were analyzed, no significant haplotype was found.

DISCUSSION

The influence of AGT genotype on pulmonary function tests in IPF

Given that diffusing capacity for carbon monoxide is the best noninvasive clinical measure of the thickness of the alveolar-capillary diffusion barrier, it was theorized that diffusing capacity would be decreased the most in individuals with AGT genotypes already associated with hypertension and/or higher rates of AGT transcription in other organs. In males with IPF, this proved to be the case; the lowest KCO values were observed in individuals with the genotypes CC at -20 and AA at -6. The most drastic decrease was observed with the CC genotype at -20, with which the KCO decreased more than two-fold compared to the AA genotype (table 5). In males, FEV_1 and FVC also increased, rather than decreased, with the CC genotype at the -20 locus; this might be due to more forceful expirations assisted by the increased elastic recoil imparted by the fibrotic lung parenchyma. Unfortunately, it is not possible to explore this hypothesis further with the LTRC dataset. Regardless, in females with IPF the lowest FVC and V_{alv} values and highest KCO were observed in individuals with the AA genotype at -6 (table 6). These data are consistent with our earlier observations [21].

Influence of gender on the effects of the -20 and -6 loci on PFTs in IPF

IPF is known to affect more men than women, but little is known about the cause of this gender difference. This study is the first to report an association with gender of genetic variants in AGT at the -20 and -6 loci at both the genotype and haplotype level. On the genotype level, the male gender had a stronger effect at the -20 locus, while the female gender imparted a greater effect at the -6 locus. Other authors studying non-pulmonary systems have also observed gender-specific effects of AGT variants; for example, Chapman et al. demonstrated that the -6 locus was also more significantly associated with increased carotid intimal medial thickening in the female population [25]. In the present study, haplotype analysis revealed that the IPF "risk haplotype" CA was significant only in males (the AG haplotype was also significant but to a lesser degree). Although other authors have noted an additional, albeit rare, SNP in AGT at the -18 position [14]. The -18 locus was genotyped here but was not analyzed further due to the lack of this variant in the LTRC or Spanish cohorts.

AGT promoter sequence variants and transcription rate

In studies of AGT synthesis by isolated human hepatocytes, SNPs at the -20 and -6 loci influence the transcription rate of AGT mRNA [14-15]. The transcription rate is higher with

the C allele at -20 and the A allele at -6. In earlier studies of both animal models of lung fibrosis and isolated lung cells, transcription of the AGT gene has been shown to be required for the fibrogenic response to bleomycin and for the apoptotic response of alveolar epithelial cells to a number of profibrotic stimuli [9,12]. Taken together, these findings suggest, and indeed had lead us to hypothesize, that higher rates of AGT transcription in lung cells imparted by the CA haplotype would lead to worse lung fibrosis in IPF patients as indicated by reductions in KCO, DLCO or FVC. As discussed above, most of these effects were found in this study, but in a surprising gender-dependent manner.

Possible mechanisms underlying gender-specific effects of AGT sequence variants

Hormonal regulatory elements located in the same AGT promoter domain as the SNPs studied here also influence the transcription rate of AGT. Of particular interest is the estrogen response element that is located in the AGT promoter region spanning – 11 to – 25 [26]. Estrogen receptor alpha (ER-α) preferentially binds to the -20 locus if the A nucleotide is present, and induces an increase in AGT transcription by human liver cells [26]. Estrogen also mediates fibrogenesis by up-regulating the transcription of procollagen I and TGF-β_1 [27]. TGF-β_1 stimulates fibroblasts to transition into myofibroblasts, which in turn deposit collagen and express AGT constitutively [9, 13]. Another potential mechanism that might regulate AGT differentially by gender is the possibility that estrogen receptor binding to the AGT promoter prevents the binding of other transcription factors that might otherwise up- or down-regulate AGT transcription.

Conversely, the binding domain of the orphan receptor Arp-1 shares homology to the binding domain for ER-α [28]. The binding of Arp-1 to this domain reduces estrogen-induced AGT transcription [28]. These data suggest that the balance between estrogen and Arp-1 at the -20 locus may thus be an influential factor in this gender discrimination. In males, it is possible that the balance may favor estrogen-induced AGT transcription instead of repression by Arp-1. IPF affects people in their fifth to eighth decade of life, and women in these decades tend to be in the post-menopausal stage. In this stage estrogen levels drop, and this may explain the bias for males at the -20 locus. In males with IPF, the KCO decreased with the presence of CC genotype at -20, while in females there was an increase in the KCO at this same locus. Thus, the balance between ER-α and Arp-1 may play a role in this difference, and this topic will be an interesting issue for future investigation.

Another possible explanation for this gender difference is the potential role of androgens in AGT transcription. Throughout the human lifespan, androgen receptors (AR) are expressed in both mesenchymal and epithelial cells. In studies of the prostate gland, ANGII enhanced the expression of AR through the ANGII type-1 receptor [29], and one of the down-stream effects of this cascade is prostate cell proliferation. If this model is applicable to the lung, modulation of androgen receptors might also contribute to increased severity of IPF in males. For these reasons, the potential role of androgens in the gender differences that AGT variants exert on IPF severity will also be an interesting topic for further research.

In this regard it is important to note that if comparisons are made of human, mouse and rat AGT promoter sequences, there is relatively low homology between these species in the TGF-β1-responsive domain of AGT between the TATA box and the transcription initiation

site [13]. Due to these sequence differences, human lung cells in culture should remain an important model to complement and extend the studies reported here. Moreover, caution should be exercised in attempts to extrapolate data on the regulation of AGT expression obtained from animal models to human lung fibrosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by the National Institutes of Health Public Health Services Grant PHS HL-45136 (M.T.D. and B.U.) and by National Science Foundation grant DMS-1209112 (C.G. and Y.C.).

REFERENCES

1. King TE, Pardo A, Selman M. Idiopathic pulmonary fibrosis. Lancet. 2011 doi: 10.1016/20140-6736(11)60052-4.
2. Datta A, Scotton CJ, Chambers RC. Novel therapeutic approaches for pulmonary fibrosis. Brit J Pharmacol. 2011; 163:141–172. [PubMed: 21265830]
3. Nicholson AG, Fulford LG, Colby TV, du Bois RM, Hansell DM, Wells AU. The relationship between individual histologic features and disease progression in idiopathic pulmonary fibrosis. Am J Resp Crit Care Med. 2002; 166:173–177. [PubMed: 12119229]
4. Weber KT, Sun Y. Recruitable ACE and tissue repair in the infracted heart. J Renin Angiotensin Aldosterone Syst. 2000; 1:295–303. [PubMed: 11967815]
5. Yoshiji H, Kuriyama S, Yoshii J, Ikenaka Y, Noguchi R, Nakatani T, Tsujinoue H, Fukui H. Angiotensin-II type I receptor interaction is a major regulator for liver fibrosis development in rats. Hepatology. 2001; 34:745–7501. [PubMed: 11584371]
6. Klahr S, Morrissey JJ. Comparative study of ACE inhibitors and angiotensin II receptor antagonists in interstitial scarring. Kidney Int Suppl. 1997; 63:S111–S114. [PubMed: 9407436]
7. Ko SH, Hong OK, Kim JW, Ahn YB, Song KH, Cha BY, Son HY, Kim MJ, Jeong IK, Yoon KH. High glucose increases extracellular matrix production in pancreatic stellate cells by activating the renin-angiotensin system. J Cell Biochem. 2006; 98:343–355. [PubMed: 16408293]
8. Marshall RP, Gohlke P, Chambers RC, Howell DC, Bottoms SE, Unger T, McAnulty RJ, Laurent GJ. Angiotensin II and the fibroproliferative response to acute lung injury. Am J Physiol Lung Cell Mol Physiol. 2004; 286:L156–L164. [PubMed: 12754187]
9. Uhal BD, Kim JK, Li X, Molina-Molina M. Angiotensin-TGF-β1 crosstalk in human idiopathic pulmonary fibrosis: autocrine mechanisms in myofibroblasts and macrophages. Curr Pharm Des. 2007; 13:1247–1256. [PubMed: 17504233]
10. Wang R, Ibarra-Sunga O, Verlinski L, Pick R, Uhal BD. Abrogation of bleomycin-induced epithelial apoptosis and lung fibrosis by captopril or by a caspase inhibitor. Am J Physiol Lung Cell Mol Physiol. 2000; 279:L143–L151. [PubMed: 10893213]
11. Li X, Rayford H, Uhal BD. Essential roles for angiotensinogen receptor AT1a in belomycin-induced apoptosis and lung fibrosis in mice. Am J Pathol. 2003; 163:2523–2530. [PubMed: 14633624]
12. Li X, Zhuang J, Rayford H, Zhang H, Shu R, Uhal BD. Attenuation of bleomycin-induced pulmonary fibrosis by intratracheal administration of antisense oligonucleotides against angiotensinogen mRNA. Curr Pharm Des. 2007; 13:1257–1268. [PubMed: 17504234]
13. Abdul-Hafez A, Shu R, Uhal BD. JunD and HIF-1α mediate transcriptional activation of angiotensinogen by TGF-β1 in human lung fibroblasts. FASEB J. 2009; 23:1655–1662. [PubMed: 19211927]
14. Yanai K, Saito T, Hirota K, Kobayashi H, Murakami K, Fukamizu A. Molecular variation of the human angiotensinogen core promoter element located between the TATA box and transcription

initiation site affects its transcriptional activity. J. Biol Chem. 1997; 272:30558–30562. [PubMed: 9374551]

15. Inoue I, Nakajima T, Williams CS, Quackenbush J, Puryear R, Powers M, Cheng T, Ludwig EH, Sharma AM, Hata A, Jeunemaitre X, Lalouel JM. A nucleotide substitution in the promoter of human angiotensinogen is associated with essential hypertension and affects basal transcription in vitro. J Clin Invest. 1997; 99:1786–1797. [PubMed: 9120024]
16. Goto S, Narita I, Saito N, Watanabe Y, Yamazaki H, Sakatsume M, Shimada H, Nishi S, Ueno M, Akazawa K, Arakawa M, Gejyo F. A(-20)C polymorphism of the angiotensinogen gene and progression of IgA nephropathy. Kidney Int. 2002; 62:980–985. [PubMed: 12164881]
17. Powell EE, Edwards-Smith CJ, Hay JL, Clouston AD, Crawford DH, Shorthouse C, Purdie DM, Jonsson JR. Host genetic factors influence disease progression in chronic hepatitis. Hepatology. 2000; 31:828–833. [PubMed: 10733535]
18. Dixon JB, Bhathal PS, Jonsson JR, Dixon AF, Powell EE, O'Brien PE. Profibrotic polymorphisms predictive of advanced liver fibrosis in the severely obese. J Hepatol. 2003; 39:967–971. [PubMed: 14642613]
19. Xiao F, Wei H, Song S, Li G, Song C. Polymorphisms in the promoter region of the angiotensinogen gene are associated with liver cirrhosis in patients with chronic hepatitis B. J Gastro and Hepatol. 2006; 21:1488–1491.
20. Wu SJ, Chiang FT, Chen WJ, Liu PH, Hsu KL, Hwang JJ, Lai LP, Lin JL, Tseng CD, Tseng YZ. Three single-nucleotide polymorphisms of the angiotensinogen gene and susceptibility to hypertension: single locus genotype vs. haplotype analysis. Physiol. Genomics. 2004; 17:79–86. [PubMed: 14970360]
21. Molina-Molina M, Xaubet A, Li X, Abdul-Hafez A, Friderici K, Jernigan K, Fu W, Ding Q, Pereda J, Serrano-Mollar A, Casanova A, Rodriguez-Becerra E, Morell F, Ancochea J, Picado C, Uhal BD. Angiotensinogen -6G>A polymorphism influences idiopathic pulmonary fibrosis disease progression. Eur Respir J. 2008; 32:1004–1008. [PubMed: 18508830]
22. Jeunemaitre X. Haplotypes of angiotensinogen in essential hypertension. Am J Hum Genet. 1997; 60:1448–1460. [PubMed: 9199566]
23. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure of extracting DNA from human nucleated cells. Nucleic Acids Res. 1988; 16:1215. [PubMed: 3344216]
24. Morgan L, Crawshaw S, Baker PN, Broughton-Pipkin F, Kalsheker N. Polymorphism in oestrogen response element associated with variation in plasma angiotensinogen concentrations in healthy pregnant women. J Hyperten. 2000; 18:553–557.
25. Chapman CML, Palmer LJ, McQuillan BM, Hung J, Burley J, Hunt C, Thompson PL, Beilby JP. Polymorphisms in the angiotensinogen gene are associated with carotid intimal-medial thickening in females from a community-based population. Atherosclerosis. 2001; 159:209–217. [PubMed: 11689223]
26. Zhao YY, Zhou J, Narayanan CS, Cui Y, Kumar A. Role of C/A polymorphism at -20 on the expression of human angiotensinongen gene. Hypertension. 1991; 33:108–115. [PubMed: 9931090]
27. Gharaee-Kermani M, Hatano K, Nozaki Y, Phan SH. Gender-based differences in bleomycin-induced pulmonary fibrosis. Am J Pathol. 2005; 166:1593–1606. [PubMed: 15920145]
28. Narayanan CS, Cui Y, Zhao YY, Zhou J, Kumar A. Orphan receptor Arp-1 binds to the nucleotide sequence located between TATA box and transcriptional initiation site of the human angiotensinogen gene and reduces estrogen induced promoter activity. Mol Cell Endocrinol. 1999; 148:79–86. [PubMed: 10221773]
29. Hoshino K, Ishiguro H, Teranishi J, Yoshida S, Umemura S, Kubota Y, Uemura H. Regulation of androgen receptor expression through angiotensin II type 1 receptor in prostate cancer cells. The Prostate. 2011; 71:964–975. [PubMed: 21541973]

Table 1

Mean values for variables of interest in the LTRC population and Spanish cohorts.

	LTRC		SPANISH	
CHARACTERISTICS[2]	**MALES (n = 94)**	**FEMALES (n = 55)**	**MALES (n = 123)**	**FEMALES (n = 80)**
AGE (years)	63.4 ± 8.5	62.4 ± 9.2	66.1 ± 10.6	67.5 ± 13.1
FEV_1 (L)	2.3 ± 0.6	1.7 ± 0.5	78.6 ± 20.0*	80.2 ± 22.5*
FVC (L)	2.8 ± 0.8	2.0 ± 0.6	70.1 ± 15.7*	71.4 ± 20.1*
FEV_1/FVC (%)	82.7 ± 6.4	83.4 ± 7.2	------	------
FEV_6 (L)	2.8 ± 0.7	2.0 ± 1.0	------	------
PEF ($L \cdot s^{-1}$)	8.5 ± 2.2	6.0 ± 1.6	------	------
TLC (% predicted)	------	------	70.5 ± 14.1	69.8 ± 15.7
PAO (mm Hg)	------	------	71.7 ± 12.8	73.3 ± 14.1
DLCO ($mL \cdot min^{-1} \cdot mmHg^{-1}$)	12.1 ± 4.7	10.0 ± 3.6	56.8 ± 16.2*	56.6 ± 18.3*
V_{alv} (L)	3.9 ± 0.9	3.0 ± 0.6		
KCO ($mL \cdot min^{-1} \cdot mmHg^{-1} \cdot L^{-1}$)	3.1 ± 0.9	3.3 ± 0.9	78.4 ± 21.0*	77.4 ± 21.3*

[1] Data are presented as mean ± SD.

[2] Abbreviations are as follows: FEV_1 = forced expiratory volume in one second; FVC = forced vital capacity; FEV_6 = forced expiratory volume in six seconds; PEF = peak expiratory flow; ; TLC = total lung capacity; PAO = alveolar-arterial oxygen tension difference; DLCO = diffusing capacity of the lung for carbon monoxide; V_{alv} = alveolar volume; KCO = ratio between DLCO and alveolar volume. 1 mm Hg = 0.133 kPa.

* Units are defined as % predicted.

Table 2

Genotype frequencies for AGT polymorphisms at -20A>C and -6G>A in the LTRC and Spanish cohorts.[1]

		LTRC			SPANISH		
SNP	**GENOTYPE**	**MALES % (94)**	**FEMALES % (55)**	**TOTAL % (149)**	**MALES % (123)**	**FEMALES % (80)**	**TOTAL % (203)**
-20A>C	**AA**	71.3 (67)	69.1 (38)	70.5 (105)	62.6 (77)	70.0 (56)	65.5 (133)
	AC	26.6 (25)	27.3 (15)	26.9 (40)	33.3 (41)	28.8 (23)	31.5 (64)
	CC	2.1 (2)	3.6 (2)	2.7 (4)	4.1 (5)	1.3 (1)	3.0 (6)
-6G>A	**GG**	28.7 (27)	29.1 (16)	28.9 (43)	28.4 (35)	33.8 (27)	30.5 (62)
	AG	59.6 (56)	50.9 (28)	56.4 (84)	52.3 (65)	47.5 (38)	50.7 (103)
	AA	11.7 (11)	20.0 (11)	14.8 (22)	18.7 (23)	18.8 (15)	18.7 (38)

[1] Data are presented as % (number of individuals from population as categorized by column heading).

Table 3

Allele frequencies for AGT polymorphisms at -20A>C and -6G>A in the LTRC and Spanish cohorts.[1]

		LTRC COHORT			SPANISH COHORT		
SNP	**ALLELE**	**MALES % (n)**	**FEMALES % (n)**	**TOTAL % (n)**	**MALES % (n)**	**FEMALES % (n)**	**TOTAL % (n)**
-20A>C	**A**	84.6 (159)	82.7 (91)	83.9 (250)	79.2 (195)	84.4 (135)	81.3 (330)
	C	15.4 (29)	17.3 (19)	16.1 (48)	20.7 (51)	15.6 (25)	18.7 (76)
-6G>A	**G**	58.5 (110)	54.6 (60)	57.1 (170)	54.9 (135)	57.5 (92)	55.9 (227)
	A	41.5 (78)	45.4 (50)	42.9 (128)	45.1 (111)	42.5 (68)	44.1 (179)

[1] Data are presented as % (number of individuals from population as categorized by column heading).

Table 4

Mean values for pulmonary function tests in the whole population at the -20 and -6 loci in the LTRC and Spanish cohorts.[1]

-20 AGT SNP in LTRC Cohort					
PHENOTYPE	**AA**	**AC**	**CC**	**P-VALUE**	**GENDER EFFECT P-VALUE**
FEV_1 (L)	2.04 ± 0.54	2.11 ± 0.76	1.93 ± 0.96	0.019 (M1)	0.0056
KCO ($mL \cdot min^{-1} \cdot mmHg^{-1} \cdot L^{-1}$)	3.25 ± 0.83	3.08 ± 0.97	2.62 ± 1.44	0.0094 (M2)	0.0023
-20 AGT SNP in Spanish Cohort					
PHENOTYPE	**AA**	**AC**	**CC**	**P-VALUE**	**GENDER EFFECT P-VALUE**
KCO (% predicted)	80.6 ± 20.0	75.8 ± 20.7	74.0 ± 8.9	0.04 (M3)	0.048
-6 AGT SNP in LTRC Cohort					
------	------	------	------	------	------
-6 AGT SNP in Spanish Cohort					
PHENOTYPE	**GG**	**GA**	**AA**	**P-VALUE**	**GENDER EFFECT P-VALUE**
DLCO (% predicted)	58.5 ± 15.2	55.5 ± 15.2	58.8 ± 15.2	0.009 (M2)	0.0031
KCO (% predicted)	79.7 ± 18.4	78.2 ± 20.8	79.4 ± 21.0	0.027 (M2)	0.0074

[1] Data are presented as mean ± SD. Significant results are accepted with $p < 0.025$ (with Bonferroni correction).

Table 5

Mean values for pulmonary function tests in the male population at the -20 and -6 loci in the LTRC and Spanish cohorts.[1]

-20 AGT SNP in male LTRC Cohort				
PHENOTYPE	**AA**	**AC**	**CC**	**P-VALUE**
FEV_1 (L)	2.21 ± 0.52	2.47 ± 0.65	2.75 ± 0.21	0.0217 (M1)
FEV_6 (L)	2.65 ± 0.55	2.95 ± 0.78	3.45 ± 0.35	0.012 (M1)
FVC (L)	2.71 ± 0.64	2.99 ± 0.56	3.45 ± 0.07	0.019 (M1)
KCO ($mL \cdot min^{-1} \cdot mmHg^{-1} \cdot L^{-1}$)	3.19 ± 0.84	3.04 ± 0.96	1.46 ± 0.34	0.0028 (M1)
-20 AGT SNP in male Spanish Cohort				
PHENOTYPE	**AA**	**AC**	**CC**	**P-VALUE**
DLCO (% predicted)	57.4 ± 12.3	52.5 ± 16.9	51.6 ± 7.1	0.05 (M3)
KCO (% predicted)	81.3 ± 20.4	72.0 ± 21.2	73.4 ± 9.8	0.017 (M3)
-6 AGT SNP in male LTRC Cohort				
	GG	**GA**	**AA**	**P-VALUE**
KCO ($mL \cdot min^{-1} \cdot mmHg^{-1} \cdot L^{-1}$)	3.23 ± 0.85	3.06 ± 0.96	3.05 ± 1.1	0.021 (M3)
-6 AGT SNP in male Spanish Cohort				
PHENOTYPE	**AA**	**AC**	**CC**	**P-VALUE**
------	------	------	------	------

[1] Data are presented as mean ± SD. Significant results are accepted with $p < 0.025$ (with Bonferroni correction).

Table 6

Mean values for pulmonary function tests in the female population at the -20 and -6 loci in the LTRC and Spanish cohorts.[1]

-20 AGT SNP in female LTRC and Spanish Cohort				
PHENOTYPE	**AA**	**AC**	**CC**	**P-VALUE**
------	------	------	------	------
-6 AGT SNP in female LTRC Cohort				
	GG	**GA**	**AA**	**P-VALUE**
FVC (L)	2.08 ± 0.58	2.19 ± 0.50	1.38 ± 0.33	0.0082 (M2)
V_{ALV} (L)	3.27 ± 0.54	3.20 ± 0.54	2.50 ± 0.69	0.022 (M2)
-6 AGT SNP in female Spanish Cohort				
	GG	**GA**	**AA**	**P-VALUE**
DLCO (% predicted)	58.1 ± 15.9	56.4 ± 17.3	69.1 ± 13.4	0.011 (M2)
KCO (% predicted	77.7 ± 18.2	78.4 ± 20.2	90.6 ± 15.0	0.023 (M2)

[1] Data are presented as mean ± SD. Significant results are accepted with $p < 0.025$ (with Bonferroni correction).