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Polymorphism in the Angiotensin II Type 1 Receptor (*AGTR1*) is Associated with Age at Diagnosis in Pulmonary Arterial Hypertension

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Abstract

Background—Pulmonary arterial hypertension (PAH) is a rare, lethal disease associated with single gene disorders, connective tissue disease, exposures to anorexigens, and often idiopathic etiology. There is evidence that genes can modify the risk of PAH: 1) monogenic disorders associated with PAH are incompletely penetrant, and 2) not all patients with associated conditions at increased risk for PAH develop the disease. The renin angiotensin aldosterone system (RAAS) provides a set of candidate genes that could modulate pulmonary vascular disease similar to its effects on renal and peripheral vasculature.

Methods—We studied 247 subjects with PAH (177 subjects with idiopathic PAH (IPAH); 63 subjects with PAH/connective tissue disease (CTD); and 7 subjects with PAH associated with anorexigens). Subjects were genotyped for five common polymorphisms in angiotensinogen (*AGT*), angiotensin converting enzyme (*ACE*), cardiac chymase A (*CMA1*), angiotensin II type 1 receptor (*AGTR1*) and aldosterone synthase (*CYP11B2*). Genotypes were tested for associations with age at diagnosis, hemodynamic parameters at diagnosis, and/or response to acute pulmonary vasodilator testing at diagnosis.

Results—Associations were demonstrated for *AGTR1* and age at diagnosis in IPAH (p=0.005). Homozygotes for the C1166 allele (n=13) were associated with an age at diagnosis 26 years later than subjects with A/A (n=139) or A/C (n=90) genotypes. No associations were demonstrated for *AGT*, *ACE, CMA1,* or *CYP11B2.*

Conclusions—The 1166C polymorphism in *AGTR1* appears to be associated with a later age at diagnosis in IPAH suggesting that this pathway could be involved in the biologic variability that is known to occur in PAH.

Keywords

genetic; pulmonary hypertension; renin; angiotensin; aldosterone

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Introduction

Pulmonary arterial hypertension (PAH) is a rare disease characterized by distinctive changes in pulmonary arterioles that lead to progressive elevation of pulmonary vascular resistance and pulmonary arterial pressure causing right ventricular failure and death if untreated (1). Histopathological studies demonstrate extensive pulmonary vascular remodeling, with intimal proliferation, smooth muscle hypertrophy, *in situ* thrombosis, fibrosis, and plexiform lesions (2). PAH is classified by etiology into idiopathic PAH (IPAH), familial PAH (FPAH), PAH associated with: connective tissue diseases (PAH/CTD), congenital systemic to pulmonary shunts (PAH/CHD), HIV-infection, drugs and toxins, portal hypertension, and persistent pulmonary hypertension of the newborn (3). Despite, recent advances in medical care, PAH still carries a poor prognosis if untreated (4) although outcome has improved with newer therapeutic drugs (5).

Mutations in three genes have been described in patients with FPAH: Bone morphogenetic protein receptor 2 (*BMPR2*)(6,7), activin receptor-like kinase-1 (*ALK1*)(8), and endoglin (*ENG*)(9), all members of the TGF-β superfamily. *BMPR2* mutations have also been reported in PAH/CHD (10) and in PAH associated with appetite suppressants (11) but not in PAH/CTD (12). Activation of the TGF-β cascade may suppress proliferation and induce apoptosis in normal pulmonary artery smooth muscle cells (13). In contrast, dysfunction of this cascade may promote an abnormal response to proliferative and apoptotic signaling factors such as cytokines, growth factors, or vascular injury thought to underlay the pathobiology in PAH. For each of the three monogenic forms of FPAH, there is incomplete penetrance with ~10-20% of *BMPR2* mutation carriers developing PAH (14). Furthermore, only a minority of patients with CTD, CHD, HIV infection, and/or exposure to anorexigens develop PAH suggesting that there are other genes that interact with *BMPR2*, *ALK1*, *ENG*, and/or environmental exposures to determine the biologic variability of PAH, e.g. penetrance, age at diagnosis, and disease severity.

The pulmonary vascular endothelium appears to play a crucial role in the pathobiology in PAH (15). Imbalances in vasoactive mediators in the lungs such as increased thromboxane and endothelin-1, and decreased prostacyclin and nitric oxide favor vasoconstriction and proliferation of smooth muscle cells (16-18). Additional factors have also been suggested such as serotonin, platelet derived growth factor, and angiotensin that could contribute to the pulmonary vasoconstriction, increased smooth muscle growth, and increased matrix deposition. The deletion/insertion polymorphism of the *angiotensin converting enzyme*(*ACE*) has been associated with IPAH and right ventricular function (19), chronic thromboembolic PH (20) and congenital diaphragmatic hernia associated with persistent pulmonary hypertension of the newborn (21). Increased *ACE* expression has also been observed in plexiform lesions in PAH (22) and, ACE inhibition appears to delay pulmonary vascular neointimal formation (23). Therefore, the Renin-Angiotensin-Aldosterone-System (RAAS) represents a group of candidate genes within a pathway that may modify disease severity and/or age at diagnosis in individuals predisposed to develop PAH. Angiotensin I is produced by cleavage of angiotensinogen (AGT) by renin and is then converted to angiotensin II predominantly by angiotensin-converting enzyme (ACE) and partly by chymase (CMA1). Angiotensin II binds primarily to the angiotensin type 1 receptor (AGTR1) to promote vascular smooth muscle constriction. Angiotensin II also acts on the adrenal cortex to promote aldosterone production by aldosterone synthase (CYP11B2). The presence of common genetic variants, or polymorphisms, in genes within the RAAS enhances RAAS activation and/or receptor function and may modify the risk of developing PAH.

In this study we analyzed five frequent polymorphisms for RAAS genes (previously shown to be associated with systemic hypertension) in subjects with IPAH, PAH/CTD, and PAH associated with anorexigens and demonstrated an association of polymorphisms in *AGTR1* with age at diagnosis in PAH. These data suggest involvement of *AGTR1* in the pathobiology of PAH and suggest novel therapeutic strategies.

Methods

We studied consecutive pediatric and adult patients referred to the New York Presbyterian Pulmonary Hypertension Center in whom the diagnosis of IPAH, PAH/CTD or PAH-anorexigen exposure was confirmed according to the WHO Venice 2003 PAH Symposium consensus. Subjects with HIV, portal hypertension, CHD, or illicit drug use were excluded due to the small number of patients available for analysis. Blood samples for genetic studies and detailed family histories were obtained in all patients. Patients were screened for mutations in *BMPR2* by dideoxy sequencing. Patients with a family history of PAH or an identified mutation in *BMPR2* were excluded due to the heterogeneity of mutations in *BMPR2* that may influence age at diagnosis and disease severity at the time of diagnosis. Two hundred and forty seven patients with PAH were included in this study (177 patients with IPAH; 63 patients with PAH/CTD; and 7 patients with anorexigen exposure). Patient characteristics are summarized in Table 1. The majority of patients were female (68% of IPAH, 100% of anorexigens, and 78% of PAH/CTD) consistent with the increased female prevalence in PAH. The majority of patients were Caucasian (79% of IPAH, 100% of anorexigens, and 73% of PAH/CTD). The mean age of IPAH patients (35 +/− 20 years mean +/− standard deviation) was younger than patients who had taken anorexigens (56 +/− 8 years) or PAH/CTD patients (59 +/− 13 years) (p=0.001). All patients had right heart catheterization and 80% of patients had acute vasodilator testing (AVT) at the time of diagnosis. Pulmonary fibrosis in PAH/CTD was excluded by pulmonary function tests, high resolution chest computed tomography and chest xray. All patients provided written informed consent (and assent if indicated) according to a protocol approved by the Institutional Review Board of Columbia University.

Hemodynamic Parameters and Acute Vasoreactivity Testing (AVT)

Standard hemodynamic parameters were obtained from right heart catheterization confirming the diagnosis of PAH at baseline on all patients and during acute vasodilator testing (AVT) with inhaled nitric oxide (80 ppm) (24). Acute pulmonary vasoreactivity was defined using the definition that appears to best predict long-term response to calcium channel blockade in adult patients. Patients who were considered acutely responsive had a fall in PAPm of ≥10 mmHg to a PAPm ≤40 mmHg with no change or an increase in cardiac output; for patients with PAPm ≤40 mmHg at rest, a decrease in PAPm and PVRi ≥20% were considered responsive to AVT. If the patient was diagnosed prior to evaluation at our center, we used the date the diagnosis was confirmed by right heart catheterization.

Molecular Analysis

Genomic DNA was isolated from whole blood using PureGene kits according to manufacturer's instructions (Gentra Systems).

Candidate gene polymorphisms in the RAAS neuro-hormonal axis were selected based on previous association studies and known functional effects of the polymorphisms (25-30). All subjects were genotyped for five RAAS gene polymorphisms: 1) a M235T missense variant angiotensinogen (*AGT*) (rs11568053), 2) a 287 base pair intron 16 deletion variant of *ACE*, 3) an A/G polymorphism at position –1903 of cardiac chymase A (*CMA1*) (rs1800875), 4) an A/C substitution at position 1166 in the 3′ untranslated region of angiotensin II type 1

receptor (*AGTR1*) (rs5186), and 5) a C/T polymorphism at position –344 in aldosterone synthase (*CYP11B2*) (rs1799998).

Polymerase chain reactions (PCR) with 100 ng of each PCR primer (supplemental Table 1) at an annealing of 55°C was used for amplification of DNA fragments. Pyrosequencing assays were performed for *AGTR1*, *CYP11B2*, *AGT*, and *CMA1*. Amplicons for pyrosequencing utilized a biotin labeled primer and were subsequently purified using streptavidin beads. Genotyping was performed according to the manufacturer's recommended protocol (Biotage, Uppsala, Sweden).

For the *angiotensin converting enzyme* (*ACE*) assay, PCR products were electrophoresed through a 2% agarose gel stained with ethidium bromide. Alleles were read as insertions or deletions by their respective sizes of 597 and 319 bp. Because the deletion allele is preferentially amplified, each apparent deletion homozygote was subject to a second PCR assay using the primers ACE5a and ACE5c that recognized sequences specific to the insertion sequence to ensure that apparent deletion homozygotes were not actually insertion/deletion heterozygotes.

Categorical data were analyzed using chi-square tests. Continuous data were analyzed using unpaired t-tests, ANOVA and ANCOVA. Baseline values where used as a covariate in the ANCOVA models. A Bonferroni correction was used to assess post hoc comparisons. Only those relationships that were pre-specified were assessed for significance. A significance level of 0.05 was used for all comparisons. SAS/Stat was used for all analysis.

Results

Genotypes were obtained in ≥99% of the patients for all five assays. Blinded replicate genotypes were performed on 10% of the patients and were 100% concordant. Each of the 5 loci was in Hardy Weinberg equilibrium. Allele frequencies for all 5 assays were similar to those previously reported for Caucasians (27,31).

Age at diagnosis was compared between genotypic classes using the categories IPAH, PAH/CTD, PAH-anorexigen exposure, and all three categories of PAH combined. For *AGTR1*, amongst the three PAH groups combined (n=242), patients homozygous C/C at position 1166 (n=13) had an average age at diagnosis of 20 and 21 years later than patients homozygous A/A (n=139) or heterozygous A/C (n=90), respectively ($p<0.005$) (Table 2). For the subset of patients with IPAH (n=174), subjects homozygous C/C (n=7) had an age at diagnosis of 26 years later then patients homozygous A/A (n=99) or heterozygous A/C (n=68) ($p<0.004$). Similarly, for patients with PAH associated with anorexigens (n=5), subjects homozygous C/C (n=1) had an age at diagnosis of 23 and 17 years later then patients homozygous A/A (n=1) or heterozygous A/C (n=3), respectively ($p<0.01$). There was no significant effect of *AGTR1* genotype on age of diagnosis in patients with PAH/CTD. The association with IPAH and all three groups of PAH remained significant after the Bonferroni correction for multiple testing. The results also remained significant after analyzing only the 80% of subjects for whom right heart catheterization acute vasodilator data were available.

In an attempt to further elucidate specific aspects associated with PAH and polymorphisms in the RAAS pathway, the endophenotypes of hemodynamic parameters including mean pulmonary artery pressure, pulmonary vascular resistance index, systemic arterial oxygen saturation, mixed venous oxygen saturation, cardiac index, and response to AVT obtained during right heart catheterization were analyzed for associations with the five RAAS gene polymorphisms. In patients with PAH/CTD, heterozygotes for the intron 16 deletion polymorphism in *ACE* were associated with decreased mixed venous oxygen saturation and

systemic arterial oxygen saturation (Table 3). Mixed venous oxygen saturation was 10% and 12% lower in heterozygotes compared to DD or II homozygotes, respectively ($p<0.004$). Systemic arterial oxygen saturation was 4% and 5% lower in heterozygotes (n=17) compared to DD (n=15) or II homozygotes (n=10), respectively ($p<0.01$); however, the association was not significant after the Bonferroni correction was applied. The M235T missense variant in *AGT* in the group of patients with PAH/CTD appeared to suggest a protective effect of the C allele for systemic arterial oxygen saturation (C/C: 95%; T/C: 92%; T/T: 89%: $p < 0.04$); however, this was not associated with age at diagnosis. In addition, although heterozygous carriers had the lowest pulmonary vascular resistance index (T/C: 16 U · m^2; T/T: 29 U · m^2; C/C: 25.6 U · m^2; $p<0.03$) and the highest mean systemic arterial pressure (T/C 84 mmHg T/T 80 mmHg; C/C 77 mmHg; $p<0.03$), these associations did not remain significant after applying the Bonferroni correction (Table 3).

For the -1903 polymorphism in *CMA1,* the heterozygotes had a higher cardiac index than either homozygous group for all categories of PAH, IPAH, and IPAH/anorexigens (Table 3). For all categories of PAH, heterozygotes (n=34) had a cardiac index of 3.2 l/min/m^2 versus 2.5 l/min/m^2 and 2.4 l/min/m^2 in the homozygous A/A (n=21) and G/G groups (n=26), respectively ($p<0.03$). When the analysis was limited to IPAH, the heterozygotes had a cardiac index of 3.2 l/min/m^2 (n=36) compared with 2.4 l/min/m^2 in both the homozygous A/A (n=24) and G/G (n=26) groups, respectively ($p<0.02$); however, these associations did not remain significant after application of the Bonferroni correction. For *AGTR1*, IPAH patients homozygous C/C at position 1166 (n =5) had a higher mean systemic arterial pressure (95 mm Hg) than patients homozygous A/A (n=89) (80 mmHg) or heterozygous A/C (n=60) (82 mmHg) ($p<0.04$), but this result was not significant after application of the Bonferroni correction.

There was no difference in response to AVT by genotypic class for any of the five RAAS polymorphisms in any of the subtypes of PAH or for all patients collectively.

Discussion

Five polymorphisms in the RAAS pathway were tested for association with age at diagnosis, disease severity (as assessed by hemodynamic parameters) at diagnosis, and/or response to acute vasodilator testing at diagnosis in patients with IPAH, PAH/CTD and PAH with anorexigen exposure. The 1166 polymorphism in *AGTR1* demonstrated an association in IPAH and PAH with anorexigen exposure and age at diagnosis thereby suggesting that the RAAS may be important in the pathobiology and biologic variability in PAH (in addition to its known association with systemic hypertension).

Homozygosity for the C 1166 allele of *AGTR1* was associated with an age at diagnosis of 26 years later in IPAH patients (and all 3 PAH groups combined) than patients with the A/A or A/C genotypes, thereby suggesting a protective effect. Similar trends were observed for PAH and anorexigen exposure although the numbers in these groups were much smaller, and the predominant effect was observed in IPAH. If confirmed in a larger cohort of patients with PAH and anorexigen exposure, these observations add further support to the biologic variability of age at onset, i.e. latency. Notably, no association was observed with *AGTR1* genotype and age at diagnosis in PAH/CTD patients. The ethnic distribution in the IPAH and PAH/CTD patients was similar as were the allele frequencies, making it unlikely that the differences were attributable to genetic differences between the populations. Rather, there may be different genetic modifiers that are specific to the various disease associations in PAH. The *AGTR1* C/C genotype was also associated with a higher systemic arterial pressure in patients with IPAH consistent with the younger age of these patients than the PAH/anorexigen patients and was no longer significant after controlling for age. The

AGTR1 genotype was not associated with any hemodynamic parameters or with acute response to nitric oxide. Previous genetic studies of the *AGTR1* polymorphism with systemic hypertension have been variable; *AGTR1* has been associated with an increased risk of systemic hypertension in Caucasians in some studies (32,33) but not in others (30,31,34). The A allele has also been associated with higher pulmonary capillary wedge and free hepatic venous pressures in patients with cirrhosis and portal hypertension (35). The A1166C variant is located in the 3′ untranslated region and has been associated with higher antiotensinogen II type 1 receptor responsiveness (36,37), mRNA stability and receptor expression (38) which could mechanistically contribute to its effects. AGTR1 activation stimulates signaling cascades that regulate cellular proliferation, fibrosis, smooth muscle hyperplasia, pro-inflammatory responses, and immune modulation that could contribute to development of PAH (39-42). If *AT1R* does play a role in the biologic variability in PAH disease progression, losartan, a nonpeptide AT1R antagonist, could be efficacious in the treatment of PAH. In addition, Benetos et al demonstrated greater responsiveness to increased vascular compliance among carriers of the C allele to the ACE inhibitor peridopril while the calcium channel blocker nitrendipine had the opposite effect (43). Such an association suggests that patients carrying certain genotypes could be treated with ACE inhibitors and/or respond differently to the use of calcium channel blockers for prevention and/or treatment for individuals at high risk of developing PAH.

However, the associations with hemodynamic factors and *ACE, AGT,* and *CMA1* were modest and were not significant after correcting for multiple testing. Therefore, we cannot suggest that disease severity (as assessed by hemodynamic parameters) at the time of diagnosis is associated with age at diagnosis or response to AVT unless our findings can be independently confirmed.

Limitations to our studies included: 1) The number of subjects we studied was modest; however for a rare disease such as PAH, the sample size was relatively large. 2) We studied a large number of phenotypes, thereby requiring a high level of statistical significance after correcting for multiple testing. Therefore, we strongly urge independent confirmation of our results. Our studies demonstrate an association with the 1166C allele of *AGTR1* with increased age at diagnosis for IPAH and suggest a similar association for PAH and anorexigen exposure. Because these are only associations, we have not necessarily identified the pathobiological genetic variants but potentially only a variant in the gene in linkage disequilibrium with the causal pathobiological variant. However, as the 1166 *AGTR1* variant has functional effects on *AGTR1* expression, it could turn out to be the causal variant.

In conclusion, the association of the C/C genotype at 1166 in *AGTR1* with a later age at diagnosis suggests a protective effect in IPAH (and possibly in PAH and anorexigen exposure) and may suggest a role for the RAAS in the pathobiology and disease progression in PAH.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Patient characteristics for each category of PAH and for the three PAH groups combined. Connective tissue diseases included systemic lupus erythematosus and the scleroderma spectrum of disease.

Type of PAH	Number	Gender (% Female)	Ethnicity	Age (years)	Age at diagnosis (years)	Mean pulmonary artery pressure (mmHg)	Pulmonary vascular resistance (U · m^2)	Mixed venous oxygen saturation (%)	Systemic arterial oxygen saturation (%)	Cardiac index (l/min/m^2)	SAPm (mmHg)
IPAH	177	68%	77% Cauc 4% AA 9% Asian 6% Hisp 4% Other	35 +/− 20	27 +/− 20	57 +/− 20	22 +/− 14	62 +/− 10	94 +/− 5	2.8 +/− 1.4	80 +/− 15
Anorexigens	7	100%	100% Cauc	56 +/− 8	51 +/− 9	53 +/− 23	23 +/− 13	59 +/− 12	90 +/− 9	2.5 +/− 1.0	111 +/− 21
PAH/CTD	63	78%	73% Cauc 11% AA 8% Asian 6% Hisp 2% Other	59 +/− 13	53 +/− 14	47 +/− 13	21 +/− 13	60 +/− 11	92 +/− 5	2.2 +/− 0.9	89+/− 14

Data are mean +/− standard deviation. For ethnicity Cauc=Caucasian; AA=African American; Hisp=Hispanic; O=Other/Mixed.

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Table 2

Association of 1166 polymorphism in *AGTR1* with age at diagnosis in PAH. Categories of PAH demonstrating association include three categories of PAH analyzed: collectively (all PAH), IPAH, and PAH/anorexigens. Values listed are mean age of diagnosis +/− standard deviation with the number of subjects indicated in parentheses.

Disease		**Genotypes**		**p**
	A/A	**A/C**	**C/C**	
All PAH	33 +/− 22 (139)	32 +/− 23 (90)	53 +/− 11 (13)	0.005
IPAH	25 +/− 19 (99)	25 +/− 21 (68)	51 +/− 8 (7)	0.004
Anorexigens	43 (1)	49 +/− 2 (3)	66 (1)	0.01

Table 3

Associations of polymorphisms in RAAS genes with disease severity (as assessed by hemodynamic parameters obtained by right heart catheterization). Categories of PAH include IPAH, PAH/CTD, PAH anorexigen exposure, and all 3 categories of PAH analyzed collectively. Values listed are mean +/− standard deviation with the number of subjects indicated in parentheses. None of the associations remained significant after correction for multiple testing.

Phenotype	Disease	Gene		Genotypes		p
		ACE	**DD**	**DI**	**II**	
Mixed venous oxygen saturation (%)	PAH/CTD		63 +/− 9 (15)	53 +/− 11 (17)	65 +/− 8 (10)	0.04
Systemic arterial oxygen saturation (%)	PAH/CTD		94 +/− 3 (15)	90 +/− 6 (17)	94 +/− 5 (10)	0.01
Mixed venous oxygen saturation (%)	All PAH		62 +/− 10 (72)	59 +/− 11 (81)	64 +/− 10 (42)	0.05
		AGT	**T/T**	**T/C**	**C/C**	
SAPm (mmHg)	IPAH		80 +/− 14 (47)	84 +/− 14 (67)	77 +/− 16 (39)	0.03
Systemic arterial oxygen saturation (%)	PAH/CTD		89 +/− 6 (8)	92 +/− 5 (24)	95 +/− 3 (9)	0.04
Pulmonary vascular resistance index (U · m^2)	PAH/CTD		29 +/− 13 (6)	16 +/− 8 (21)	26 +/− 19 (9)	0.03
		CMA1	**A/A**	**A/G**	**G/G**	
Cardiac index (l/min/m^2)	All PAH		2.5 +/− 1.3 (21)	3.2 +/− 1.6 (34)	2.4 +/− 0.9 (26)	0.03
	IPAH		2.4 +/− 1.2 (24)	3.2 +/− 1.6 (36)	2.4 +/− 0.9 (26)	0.02
	IPAH/anorexigens		2.4 +/− 1.2 (35)	2.9 +/− 1.4 (50)	2.3 +/− 0.9 (42)	0.03
		AGTR1	**A/A**	**A/C**	**C/C**	
SAPm (mmHg)	IPAH		80 +/− 15 (86)	82 +/− 14 (60)	95 +/− 10 (5)	0.04