article

Synergistic heterozygosity for *TGFβ1* SNPs and *BMPR2* mutations modulates the age at diagnosis and penetrance of familial pulmonary arterial hypertension

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Purpose: We hypothesized that functional *TGFβ1* SNPs increase TGFβ/BMP signaling imbalance in *BMPR2* mutation heterozygotes to accelerate the age at diagnosis, increase the penetrance and SMAD2 expression in familial pulmonary arterial hypertension. **Methods:** Single nucleotide polymorphism genotypes of *BMPR2* mutation heterozygotes, age at diagnosis, and penetrance of familial pulmonary arterial hypertension were compared and SMAD2 expression was studied in lung sections. **Results:** *BMPR2* mutation heterozygotes with least active -509 or codon 10 *TGFβ1* SNPs had later mean age at diagnosis of familial pulmonary arterial hypertension (39.5 and 43.2 years) than those with more active genotypes (31.6 and 33.1 years, $P = 0.03$ and 0.02, respectively). Kaplan-Meier analysis also showed that those with the less active single nucleotide polymorphisms had later age at diagnosis. *BMPR2* mutation heterozygotes with nonsense-mediated decay resistant *BMPR2* mutations and the least, intermediate and most active -509 *TGFβ1* SNP genotypes had penetrances of 33, 72, and 80%, respectively ($P = 0.003$), whereas those with 0–1, 2, or 3–4 active single nucleotide polymorphism alleles had penetrances of 33, 72, and 75% ($P = 0.005$). The relative expression of TGFβ1 dependent SMAD2 was increased in lung sections of those with familial pulmonary arterial hypertension compared with controls. **Conclusions:** The *TGFβ1* SNPs studied modulate age at diagnosis and penetrance of familial pulmonary arterial hypertension in *BMPR2* mutation heterozygotes, likely by affecting TGFβ/BMP signaling imbalance. This modulation is an example of Synergistic Heterozygosity. ***Genet Med* 2008:10(5):359–365.**

Key Words: *synergistic heterozygosity,* TGFβ1 *SNPs,* BMPR2 *mutations, FPAH*

Pulmonary arterial hypertension (PAH) is a progressive, fatal disease caused by proliferative occlusion of the pulmonary arteries due to vascular remodeling. Familial PAH (FPAH) is an autosomal dominant disorder that exhibits variable expression, reduced penetrance, skewed female:male ratio of 2.4:1 and anticipation.[1,2] In 2000, mutations in the Bone Morphogenetic Protein Receptor Type 2 (*BMPR2*) gene in the TGFβ Superfamily were reported to cause FPAH.[3,4] Subsequently, over 140 different *BMPR2* mutations have been detected in FPAH and most are predicted to cause a loss of receptor function (haploinsufficiency), either through missense, nonsense or frameshift mutations, or through splicing errors.[5–8] Recently, these as well as deletion/duplication *BMPR2* mutations were reported to occur in the majority (82%) of FPAH cases.[9]

Although *BMPR2* mutations cause FPAH, the pathophysiology of this complex disease remains unclear.[10] The observation that many of those with a *BMPR2* mutation do not develop FPAH suggests that susceptibility and modifier genes, anticipation, as well as environment may all play important roles in determining the reduced penetrance, skewed gender ratio, variations in clinical severity and progression, and possibly differences seen in the response to treatment.

Anticipation is the tendency in certain genetic disorders for those in successive generations to present at an earlier age and/or with more severe manifestations. It is often seen in disorders resulting from the expression of a trinucleotide repeat expansion that tends to increase in size and have a more significant effect when passed from one generation to the next.[11] No trinucleotide repeat or other expansions in *BMPR2*

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have been reported to cause FPAH and the molecular basis of anticipation in FPAH is poorly understood.[1,2]

Nonsense-mediated decay (NMD) is a messenger RNA (mRNA) surveillance system that degrades transcripts containing premature termination codons (PTCs) to prevent translation of unnecessary or harmful transcripts. We have previously shown by analysis of *BMPR2* mRNAs that some mutations produce stable transcripts whereas others generate PTCs which trigger mRNA degradation by NMD.[7,9] Failure to eliminate mRNAs with PTCs can result in synthesis of abnormal proteins that can be toxic to cells through dominant negative or gain of function effects.[12,13]

Ligands for BMPR2, called bone morphogenetic proteins (BMPs), are signaling molecules that are also members of the TGFβ superfamily. BMPs are synthesized and released from a variety of cell types including pulmonary artery smooth muscle and endothelial cells. They induce formation of heterodimeric complexes between Type I and II receptors that activate downstream signaling pathways through phosphorylation and/or mitogen activated kinases.[14,15] SMADS1, 5, and 8 mediate intracellular signaling of BMP whereas SMADS2 and 3 transduce TGFβ signaling. SMAD4 is a common mediator that is required for both pathways.[16] In addition, other signaling systems that influence vascular tone, function, and remodeling have been associated with PAH.[17–19] Multiple studies show that serotonin and its transporter (SERT) play a critical role in the pulmonary vascular smooth muscle hyperplasia and vascular remodeling found in PAH and *SERT* polymorphisms weakly correlate with severity or age at diagnosis (AAD) of FPAH.[20–22]

Susceptibility genes have functional variants that affect causes of disease. Modifier genes differ in that they are genetic variants that affect the clinical manifestation of disease (as opposed to liability). In contrast to susceptibility genes, the identification of modifier genes has proven elusive.[23] *TGFβ* single nucleotide polymorphisms (SNPs) have been reported to modify the severity of cystic fibrosis (CF).[24] Drumm et al.[24] reported significant allelic and genotypic associations between the CF phenotype and -509 and codon 10 *TGFβ1* SNPs. The -509 TGFβ C/T SNP explains 8.2% of the additive genetic variance in TGFβ concentration due to effects on a Yin Yang 1 binding site and transcriptional suppression of the -509 C allele by AP1.[25–27] The codon 10 C SNP allele with higher levels of *TGFβ1* mRNA and protein exhibits association with pulmonary fibrotic and chronic obstructive pulmonary disease.[28–30] The more active -509 TT and codon 10 CC *TGFβ1 SNP* genotypes associate in CF studies with worse lung function, and the *TGFβ1* codon 10 CC genotype had an odds ratio of 2.2 with the most severe reductions in forced expiratory volume in the first second (FEV1).[24] Although the codon 10 CC SNP genotype correlated with increased TGFβ expression, secretion and levels in a variety of other conditions, the mechanisms by which the SNP genotype may function with other genetic or environmental factors to affect the severity of CF is unknown.[23]

Variation in the severity of symptoms of inborn errors is often attributed to the effects of specific mutations. However, some affected individuals can have partial defects in more than one pathway, or at multiple steps in a single pathway. These individuals can show clinical symptoms consistent with a homozygous defect in the affected pathway even though they do not have a complete deficiency in any one enzyme. Vockley et al.[31] and Schuler et al.[32] coined the term "Synergistic Heterozygosity" for such individuals having clinically significant metabolic problems due to the compound effects of these partial defects. We have incorporated this idea of Synergistic Heterozygosity into our hypothesis that heterozygosity for both a rare *BMPR2* mutation and common *TGFβ* SNPs in the TGFβ/BMP signaling pathways interact to influence expression of the FPAH phenotype.

MATERIALS AND METHODS

Patient recruitment and evaluation

Study subjects were recruited over a 20-year period through our Pulmonary Hypertension Center, the Pulmonary Hypertension Association, and the National Institutes of Health Clinical Trials website (http://clinicaltrials.gov). The 81 family members with FPAH and a proven *BMPR2* mutation were treated at centers throughout the United States and most are deceased. These 81 individuals were members of 55 nonrelated FPAH kindreds. Of these, 40 families were represented by one subject, whereas 11 families contributed two subjects. The remaining 19 affected subjects represented four different families, with three, four, five, and seven subjects, respectively. PAH was diagnosed by lung pathology showing plexogenic pulmonary arteriopathy in the absence of alternative causes such as congenital heart disease, or by clinical and cardiac catheterization criteria. The latter included a mean pulmonary arterial pressure of $>$25 mm Hg with a pulmonary capillary or left atrial pressure of $<$15 mm Hg, and exclusion of other causes of pulmonary hypertension in accordance with accepted international standards of diagnostic criteria.[33,34] Pulmonary arterial pressures were similar, regardless of the age at diagnostic cardiac catheterization. Diagnosis of FPAH required a well-documented family history of PAH and clinical confirmation of FPAH by the above criteria. Clinical information on current age, AAD, gender, and PAH status was confirmed by review of medical records. AAD for patients with FPAH was determined by the date of diagnostic cardiac catheterization or clinical evaluation that established the diagnosis. A careful scrutiny of available clinical data including AAD resulted in the inclusion of 61 of the 81 affected subjects for the analysis of factors influencing AAD. For the 39 unaffected members of our FPAH families who also had a proven *BMPR2* mutation, their age on the date of our last contact with them at study enrollment and blood draw was recorded and their AAD was censored at the age of enrollment. Thus, all subjects in this study were proven to have *BMPR2* mutations and were classified as affected based on the presence of the FPAH diagnosis, versus mutation carriers without clinical evidence of PAH. The study was approved by the Institutional Review Board at Vanderbilt University Medical Center, and written informed consent was obtained from all study subjects.

Genotyping genomic DNAs for TGFβ SNPs

Genomic DNA was prepared from whole blood using Gentra Systems' Puregene® DNA Purification Kit (Minneapolis, MN) according to the manufacturer's protocol. Samples were genotyped for *TGFβ1* -509 and codon 10 SNPs by polymerase chain reaction (PCR) amplification of 25 ng of genomic DNA using Elongase Enzyme Mix (Invitrogen, Carlsbad, CA) and 10 μM of each of the oligonucleotide primers. The sequences for the *TGFβ1* -509 primers were 5′-CAGTTGGCGAGAACAGTTGG-3′ for the forward primer and 5′-CAGATGCGCTGTGGCTTT-3′ for the reverse primer. The sequences for the *TGFβ1* codon 10 primers were 5′-GCCACAGATCCCCTATTCAA-3′ for the forward primer, and 5′-TCGATAGTCTTGCAGGTGGA-3′ for the reverse primer. PCR cycling conditions for *TGFβ1* -509 were 94°C, 30 seconds; 35 cycles of 94°C, 30 seconds; 58°C, 45 seconds; 68°C, 45 seconds; then 68°C, 3 minutes. PCR cycling conditions for *TGFβ1* codon 10 were 95°C, 1 minute; 35 cycles of 95°C, 1 minute; 61°C, 1 minute; 68°C, 1 minute; then 68°C, 10 minutes. Before sequencing, amplified products were purified with ExoSAP-IT (USB Corporation, Cleveland, OH). Sequencing reactions were performed using a forward primer (5′-GCCCAGTTTCCCTATCTGT-3′) for *TGFβ1* -509 and another forward primer (5′-CCGTGGGATACTGAGACACC3′) for *TGFβ1* codon 10 and a BigDye Terminator v3.1 Cycle Sequencing Kit according to the manufacturer's protocol (Applied BioSystems, Foster City, CA). Samples were then analyzed by capillary electrophoresis using a 3100 Genetic Analyzer (Applied Biosystems). Genotypes were determined by analysis with Mutation Surveyor Software (SoftGenetics, State College, PA).

NMD classification

BMPR2 mutations that produce stable mutant *BMPR2* transcripts that do not activate NMD, are referred to as NMD− whereas those whose transcripts are degraded by NMD are classified as NMD+. NMD status was based on the stability of the mutant transcript in lymphoblastoid cell lines derived from individuals with each mutation following incubation with or without an NMD inhibitor (Puromycin).[7,9] All NMD− *BMPR2* transcripts showed roughly equal proportions of mutant and normal *BMPR2* transcripts with and without lymphoblastoid cell line incubation with Puromycin.

Preparation of lung sections and SMAD analysis

Immunohistochemical analysis of human tissue sections for phosphorylated SMAD2 (pSMAD2) was performed using reagents and methods that have been previously described.[35] Samples were derived from control individuals without disease (death caused by trauma) and diseased control patients with either pulmonary hypertension associated with congenital heart disease/Eisenmenger syndrome or FPAH due to mutations in the *BMPR2* gene ($n = 5$ in each group). The images were analyzed independently by two pathologists who were blinded to clinical diagnosis.

Study design

We hypothesized that Synergistic Heterozygosity for functional *TGFβ1* SNPs further increases the TGFβ/BMP signaling imbalance in *BMPR2* mutation heterozygotes (MH) to modulate the AAD and the penetrance of FPAH and is associated with increased SMAD2 expression in the lung. To test our hypotheses we genotyped the *TGFβ1* SNPs of *BMPR2* MH and compared both the AAD and penetrance of FPAH among the *TGFβ1* SNPs genotypes. We also examined the expression of TGFβ/BMP dependent SMAD2 in lung sections from individuals with FPAH.

Data analysis and statistics

The test proposed by Weir[36] was applied for Hardy Weinberg equilibrium (HWE). Kaplan-Meier disease free survival curve for AAD was constructed for *TGFβ1* SNP genotype groups. Log rank test was used to compare the curves between groups. For FPAH patients, their AAD was compared using Wilcoxon rank sum test because the AAD was not normally distributed. P values < 0.05 were considered statistically significant and all tests were two-tailed. Analysis of the binary variable penetrance was undertaken using the chi-square test for trend (Mantel-Haenszel test) across the three genotypes for each SNP. P values < 0.025, in concordance with techniques of the Bonferroni correction, were considered statistically significant and all tests were two-tailed. Statistical analyses were performed on a personal computer with the statistical package SPSS for Windows (Version 14.0, SPSS, Chicago) and the statistical software R (www.r-project.org).

RESULTS

TGFβ1 SNP genotypes

The TGFβ -509 and codon 10 SNP genotypes for affected individuals are shown in Tables 1 and 2. The P values of tests for HWE for the affected (FPAH patients) were 0.723 and <0.001 for *TGFβ* -509 and codon 10 SNP genotypes, respectively.

AAD of FPAH and *TGFβ1* SNP genotypes

BMPR2 MH having the least active (CC) as opposed to more active (CT/TT) -509 *TGFβ1* SNP genotypes had mean ages at

Table 1
TGFβ1 -509 SNP genotypes and the observed and expected allele distributions among all affected subjects

	TGFβ1 -509	Individuals observed[a]	Individuals expected
Increasing TGFβ-1 activity ↓	CC	36	39.5
	CT	41	34.1
	TT	4	7.4
	Total	81	81

[a]C = 0.698 and T = 0.302.

Table 2
TGFβ1 codon 10 SNP genotypes and the observed and expected allele distributions among all affected subjects

	TGFβ1 codon 10	Individuals observed[a]	Individuals expected
Increasing TGFβ-1 activity ↓	TT	19	26.7
	TC	55	39.6
	CC	7	14.7
	Total	81	81

[a]T = 0.574 and C = 0.426.

Table 3
Mean age at diagnosis of FPAH for different *TGFβ1* -509 SNP genotypes

	TGFβ1 -509	Age (yrs)	Individuals
Increasing TGFβ-1 activity ↓	CC	39.5	29
	CT/TT	31.6	32
	Total	35.4	61

$P = 0.03$ Wilcoxon.

Table 4
Mean age at diagnosis of FPAH for different *TGFβ1* codon 10 SNP genotypes

	TGFβ1 codon 10	Age (yrs)	Individuals
Increasing TGFβ-1 activity ↓	TT	43.2	14
	TC/CC	33.1	47
	Total	35.4	61

$P = 0.02$ Wilcoxon.

diagnosis of FPAH of 39.5 and 31.6 years, respectively ($P = 0.03$ Wilcoxon, Table 3). *BMPR2* MH with the least active (TT) as opposed to more active (CT/CC) codon 10 *TGFβ1* SNP genotypes had mean ages at diagnosis of 43.2 and 33.1 years, respectively ($P = 0.02$ Wilcoxon, Table 4).

Kaplan-Meier analyses

Kaplan-Meier analyses showed that those having the least active (CC) versus the more active (CT/TT) *TGFβ1* -509 SNP genotypes had later ages at diagnosis ($P = 0.038$ log rank, Fig. 1). Also those having the least active (TT) versus the more active (CT/CC) *TGFβ1* codon 10 SNP genotypes had later ages at diagnosis ($P = 0.020$ log rank, Fig. 1).

Penetrance of FPAH in *BMPR2* heterozygotes with *TGFβ1* SNP genotypes

The proportion of all NMD− *BMPR2* MH who had FPAH was 0.33, 0.72, and 0.80 for those with CC (least active), CT (intermediate) and TT (most active) -509 genotypes ($P = 0.003$ from χ^2 trend test, Fig. 2). The proportion of all NMD− *BMPR2* MH who had FPAH was 0.33, 0.72, and 0.75 for those with 0–1, 2, or 3–4 active -509 or codon 10 SNP alleles ($P = 0.005$ from χ^2 trend test, Fig. 3).

SMAD analysis of lung sections

When compared with control individuals, the small to medium sized muscular arteries in the pulmonary circulation of individuals with FPAH showed increased TGFβ signaling in the medial layer of the vessel wall, as evidenced by increased nuclear accumulation of phosphorylated SMAD2 (pSMAD2), an intracellular mediator of the TGFβ transcriptional response (Fig. 4). This observation is unlikely to simply reflect a consequence of a chronic increase in pulmonary arterial pressure because increased TGFβ signaling was not observed in the pulmonary vasculature of individuals with Eisenmenger syndrome, a form of secondary pulmonary hypertension due to a chronic shunting of blood flow into the pulmonary circulation from the high-pressure systemic circulation (Fig. 4).

DISCUSSION

Although FPAH is autosomal dominant it has reduced penetrance and variable age of onset in *BMPR2* MH.[1,17,18] This suggests that the genetic background on which *BMPR2* mutations occur, along with the expression levels of the mutant and normal *BMPR2* alleles, and possible environmental factors may affect penetrance and age of onset of FPAH. Identifying the genetic variations in susceptibility and modifier genes that enhance or inhibit phenotypic expression could provide insights that might allow directed intervention strategies to be developed. To better understand the role of modifier genes in FPAH we studied the relationship between variations in the number of active *TGFβ* SNPs and the AAD and penetrance of FPAH in *BMPR2* MH.

The *TGFβ1* -509 and codon 10 SNP gene frequencies were C = 0.69, T = 0.31 and C = 0.43, T = 0.57, respectively (Tables 1 and 2). From these frequencies the expected numbers of individuals of various genotypes were calculated using the Hardy Weinberg formula. Tests for HWE showed that the codon 10 genotypes deviated from HWE. To exclude the possibility of genotyping errors, we regenotyped both SNPs from fresh aliquots of all genomic DNA samples and genotyping errors were ruled out. One possible explanation for the deviation from HWE may be our study ascertainment. In this aspect of our study, every subject has a *BMPR2* mutation. Thus, our study sample comprises a stratum from the general population, rather than a representative sample. Secondly, this analysis fo-

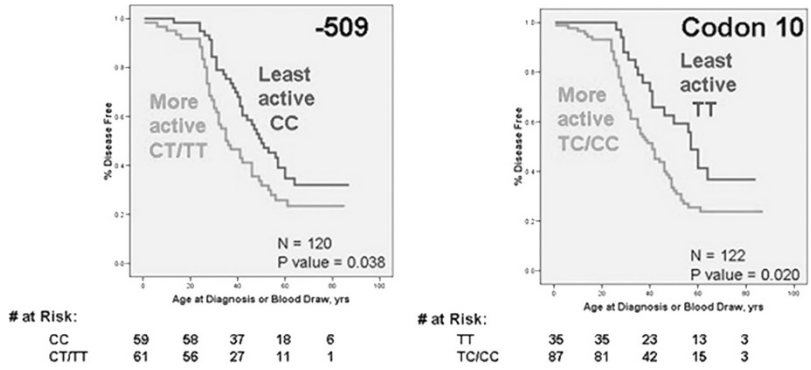

Fig. 1. Kaplan-Meier survival analysis of the age at diagnosis of FPAH or blood draw of unaffected *BMPR2* mutation heterozygotes (MH) and the number of active -509 (left) or codon 10 (right) *TGFβ1* SNP alleles.

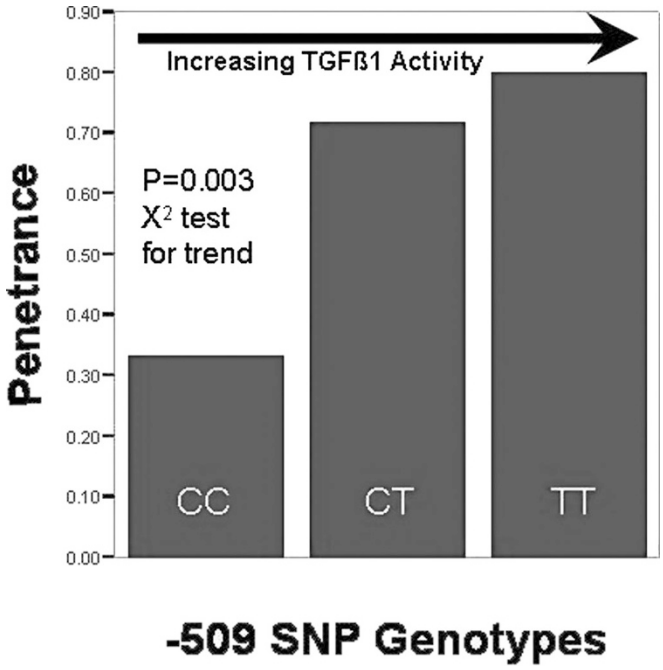

Fig. 2. Penetrance of FPAH in nonsense-mediated decay negative (NMD−) *BMPR2* MH with -509 *TGFβ1* SNP genotypes. The proportion of all NMD− *BMPR2* MH was 0.33, 0.72, and 0.80 for those with CC (least active), CT (intermediate), and TT (most active) -509 genotypes.

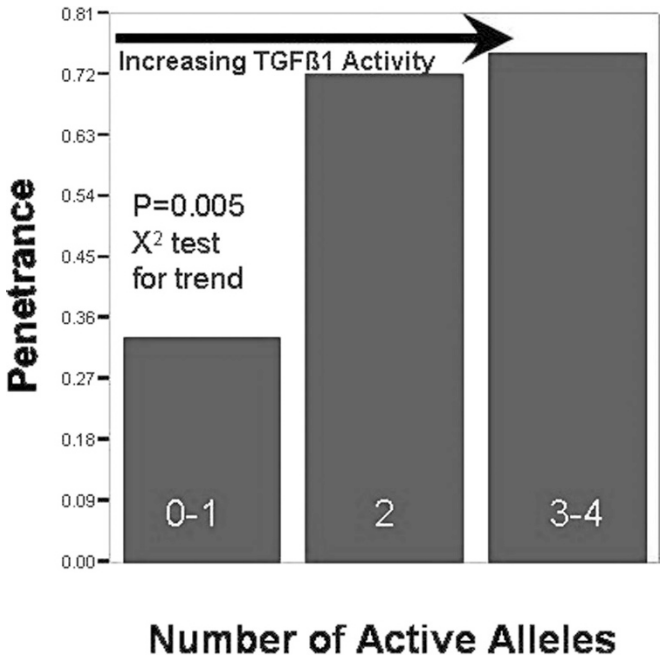

Fig. 3. Penetrance of FPAH in NMD− *BMPR2* MH with the number of -509 and codon 10 *TGFβ1* SNP alleles with increased activity. The proportion of all NMD− *BMPR2* MH who had FPAH was 0.33, 0.72, and 0.75 for those with 0–1, 2, or 3–4 active -509 or codon 10 SNP alleles, respectively.

cuses on the affected, and HWE is generally expected to be distorted in the case sample in the region of association.[37]

We hypothesized that increased TGFβ activation from more active *TGFβ* alleles, coupled with reduced BMPR2 function from heterozygosity for a *BMPR2* mutation can further increase the imbalance in the TGFβ/BMP signaling ratio to contribute to an earlier AAD and increased penetrance of FPAH. We tested our hypotheses first by studying the association of common, functional *TGFβ1* SNPs and AAD of those having FPAH (Tables 3 and 4 and Figs. 1 and 2). We also determined the penetrance of FPAH in all NMD− *BMPR2* MH with different *TGFβ* SNP genotypes (Figs. 3 and 4). Both earlier AAD and increased penetrance was seen in those with more active *TGFβ* SNP genotypes. To evaluate the potential that familial clustering may contribute to these findings, we excluded families having more than two affected individuals, and reanalyzed the data. Despite a loss of power due to the exclusion of 19 affected subjects and 17 unaffected heterozygotes, a significantly younger AAD and increased penetrance was again seen in those with more active *TGFβ* SNP genotypes. In addition, many of the remaining subjects from the 11 FPAH kindreds with two affected members were distantly related. Thus, even for the few remaining closely related members the probability for concordance for *TGFβ* SNP genotype is expected to be similar to that of the general population because of the high frequencies of the SNP alleles. Finally, when we selected only the youngest affected *BMPR2* MH from each family for which mutation and SNP data were available we observed the same affects of the SNPs on AAD (data not shown). Thus, we feel that the contribution of familial clustering is negligible.

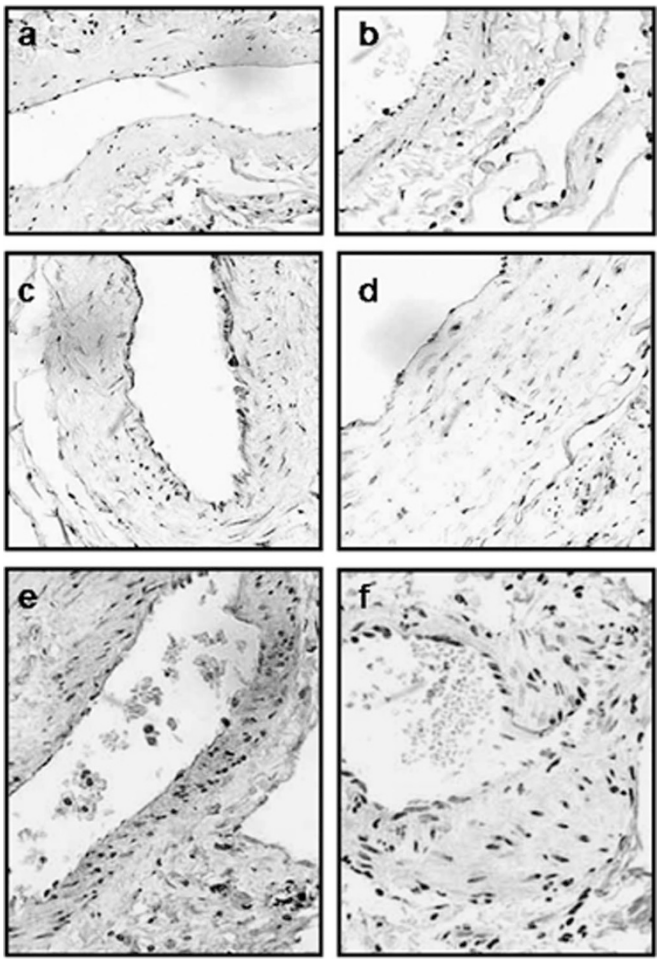

Fig. 4. TGFβ signaling, as marked by nuclear accumulation of phosphorylated SMAD2 (pSMAD2). In small to medium sized muscular arteries in the pulmonary circulation in normal lung (a, b), lung from individuals with secondary pulmonary hypertension (Eisenmenger syndrome; c, d), and lung from individuals with primary pulmonary hypertension caused by mutations in *BMPR2* (e, f). Fields are representative of those observed in five individuals in each phenotypic category. Magnification ×20 (a, c, e) and ×40 (b, d, f).

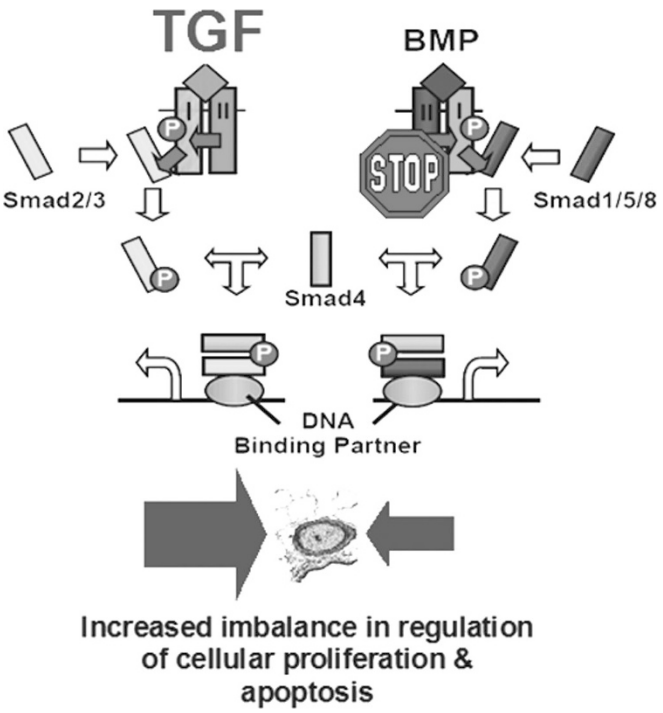

Fig. 5. Model of imbalanced TGFβ/BMP pathway signaling and Synergistic Heterozygosity for rare *BMPR2* mutations and common, functional *TGFβ1* SNPs that can cause earlier age at diagnosis, increased penetrance, and increased SMAD2 accumulation in FPAH.

Our finding that *TGFβ1* SNPs are associated with AAD and penetrance agrees with other evidence that increased TGFβ activity promotes pulmonary vascular disease in genetic association studies.[24,29,30] Multiple, common human TGFβ ligand SNPs are known to affect TGFβ activation.[25,38] In twin studies the TGFβ1 concentration was shown to be predominantly under genetic control (heritability estimate 0.54).

Also in support of our hypothesis that imbalanced TGFβ /BMP signaling due to the presence of functional *TGFβ* SNP alleles correlates with the AAD and penetrance of FPAH (Tables 3 and 4 and Figs. 1–3), we found that SMAD2, which is induced by TGFβ, shows increased relative expression in lung sections of individuals with FPAH (Fig. 4). Thus, our data in aggregate, suggest that common *TGFβ* SNPs and rare *BMPR2* mutations combine to increase the imbalance between the downstream pathways of TGFβ and BMPR2 signaling to accelerate the diagnosis of FPAH (Fig. 5).

Our data suggesting the potential role of *TGFβ1* SNPs as modifier genes for FPAH are analogous to findings in CF.[24] Importantly, our results suggest that possible clinical therapies could target the TGFβ/BMP signaling imbalance. Several potential approaches exist, which, except for Losartan in Marfan syndrome and Imatinib (Gleevec) in PAH have not been tested in humans.[39–41] Losartan attenuates pulmonary arterial remodeling and increased BMPR2 expression in a swine model.[42] Excess TGFβ1 activity is associated with Fibrillin mutations in Marfan syndrome.[43] Losartan is an angiotensin receptor blocker that reduces cleavage of TGFβ1 from its latent complex and prevents aortic aneurysms in a murine model of Marfan syndrome; it is in clinical trials in human Marfan syndrome.[44]

The mechanisms underlying genetic anticipation in FPAH have yet to be elucidated.[1,2] Our finding that the number of more active *TGFβ* SNPs correlates with AAD and penetrance suggests that polymorphic variation in *TGFβ1* may contribute to genetic anticipation. Our data indicate that *BMPR2* MH with no active *TGFβ1* -509 or codon 10 SNPs have a later AAD and constitute the minority (47 and 23%, respectively) of affected individuals studied (Tables 3 and 4). Thus, *BMPR2* MH with a later AAD are more likely to lack more active *TGFβ* SNPs. Because population data indicates that most in the normal population would have one or more active alleles, offspring who inherit the *BMPR2* mutation from an affected parent who has no active *TGFβ* SNPs would likely have one or two active *TGFβ* SNPs they inherited from their "normal " parent. Thus the child, unlike their *BMPR2* heterozygous parent, would have one or two active *TGFβ* SNPs that promote an earlier AAD and increased penetrance. Our conclusion regard-

ing the potential effects of SNP genotypes on apparent anticipation is supported by our seeing the same effects of SNPs on AAD when we analyzed only the youngest affected *BMPR2* MH from each family having available mutation and SNP data. In summary, we think this could exemplify "synergistic anticipation" in which older onset *BMPR2* heterozygotes with younger onset children lack, while their children usually possess, common *TGFβ* SNPs that are associated with earlier AAD of FPAH.

Finally, we think that the interactions between these *TGFβ* SNPs and *BMPR2* mutations create cases of double and triple heterozygosity that are examples of "Synergistic Heterozygosity."[31,32] In the case of FPAH, heterozygosity for more active TGFβ alleles is predicted to further increase the imbalance in the TGFβ/BMP signaling ratio that is already enhanced as a result of heterozygosity for a *BMPR2* mutation. In FPAH this Synergistic Heterozygosity occurs in reciprocal, interactive pathways as shown in Figure 5. It is also possible that additional, but as yet unknown, polymorphisms in other genes in the TGFβ pathway may also contribute to this synergism.

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References

1. Newman JH, Wheeler L, Lane KB, Loyd E, et al. Mutation in the gene for bone morphogenetic protein receptor II as a cause of primary pulmonary hypertension in a large kindred. *N Engl J Med* 2001;345:319–324.
2. Loyd JE, Butler MG, Foroud TM, Conneally PM, et al. Genetic anticipation and abnormal gender ratio at birth in familial primary pulmonary hypertension. *Am J Respir Crit Care Med* 1995;152:93–97.
3. Lane KB, Machado RD, Pauciulo MW, Thomson JR, et al. Heterozygous germ-line mutations in BMPR2, encoding a TGF-β receptor, cause familial primary pulmonary hypertension. The International PPH Consortium. *Nat Genet* 2000;26:81–84.
4. Deng Z, Morse JH, Slager SL, Cuervo N, et al. Familial primary pulmonary hypertension (gene PPH1) is caused by mutations in the bone morphogenetic protein receptor-II gene. *Am J Hum Genet* 2000;67:737–744.
5. Machado RD, Pauciulo MW, Thompson JR, Lane KB, et al. BMPR2 haploinsufficiency as the inherited molecular mechanism for primary pulmonary hypertension. *Am J Hum Genet* 2001;68:92–102.
6. Atkinson C, Stewart S, Upton PD, Machado R, et al. Primary pulmonary hypertension is associated with reduced pulmonary vascular expression of type II bone morphogenetic protein receptor. *Circulation* 2002;105:1672–1678.
7. Cogan JD, Vnencak-Jones CL, Phillips JA III, Lane KB, et al. Gross BMPR2 gene rearrangements constitute a new cause for primary pulmonary hypertension. *Genet Med* 2005;7:169–174.
8. Machado RD, Aldred MA, James V, Harrison RE, et al. Mutations of the TGF-β type II receptor *BMPR2* in pulmonary arterial hypertension. *Hum Mutat* 2006;27:121–132.
9. Cogan JD, Pauciulo MW, Batchman AP, Prince MA, et al. High frequency of BMPR2 exonic deletions/duplications in familial arterial hypertension. *Am J Resp Crit Care Med* 2006;174:590–598.
10. Yang X, Long L, Southwood M, Rudarakanchana N, et al. Dysfunctional Smad signaling contributes to abnormal smooth muscle cell proliferation in familial pulmonary arterial hypertension. *Circ Res* 2005;96:1053–1063.
11. Mitas M. Trinucleotide repeats associated with human disease. *Nucleic Acids Res* 1997;25:2245–2254.
12. Le Hir H, Izaurralde E, Maquat LE, Moore MJ. The spliceosome deposits multiple proteins 20–24 nucleotides upstream of mRNA exon–exon junctions. *EMBO J* 2000;19:6860–6869.
13. Le Hir H, Izaurralde E, Maquat LE. Pre-mRNA splicing alters mRNP composition: evidence for stable association of proteins at exon–exon junctions. *Genes Dev* 2000; 14:1098–1108.
14. Yamashita H, Ten Dijke P, Heldin CH, Miyazono K. Bone morphogenetic protein receptors. *Bone* 1996;19:569–574.
15. Nohe A, Hassel S, Ehrlich M, Neubauer F, et al. The mode of bone morphogenetic protein (BMP) receptor oligomerization determines different BMP-2 signaling pathways. *J Biol Chem* 2002;277:5330–5338.
16. Sakou T, Onishi T, Yamamoto T, Nagamine T, et al. Localization of Smads, the TGF-beta family intracellular signaling components during endochondral ossification. *J Bone Miner Res* 1999;14:1145–1152.
17. Newman JH, Fanburg BL, Archer SL, Badesch DB, et al. Pulmonary arterial hypertension: future directions: report of an NHLBI/ORD Workshop. *Circulation* 2004; 109:2947–2952.
18. Newman JH, Trembath RC, Morse JA, Grunig E, et al. Genetic basis of pulmonary arterial hypertension: current understanding and future directions. *J Am Coll Cardiol* 2004;43:S33–S39.
19. Morrell N. Pulmonary hypertension due to BMPR2 mutations. *Proc Am Thoracic Soc* 2006;3:680–686.
20. Eddahibi S, Hanoun N, Lanfumey L, Lesch KP, et al. Attenuated hypoxic pulmonary hypertension in mice lacking the 5-hydroxytryptamine transporter gene. *J Clin Invest* 2000;105:1555–1562.
21. Eddahibi S, Humbert M, Fadel E, Raffestin B, et al. Serotonin transporter overexpression is responsible for pulmonary artery smooth muscle hyperplasia in primary pulmonary hypertension. *J Clin Invest* 2001;108:1141–1150.
22. Willers ED, Newman JH, Loyd JE, Robbins IM, et al. Serotonin transporter polymorphisms in familial and idiopathic pulmonary arterial hypertension. *Am J Respir Crit Care Med* 2006;173:798–802.
23. Haston CK, Hudson TJ Jr. Finding genetic modifiers of cystic fibrosis. *N Engl J Med* 2005;353:1509–1511.
24. Drumm ML, Konstan MD, Schluchter MD, Handler A, et al. Genetic modifiers of lung disease in cystic fibrosis. *N Engl J Med* 2005;353:1443–1453.
25. Grainger DJ, Heathcote K, Chiano M. Genetic control of the circulating concentration of transforming growth factor type beta 1. *Hum Mol Genet* 1999;8:93–97.
26. Silverman ES, Palmer LJ, Subramaniam V, Hallock A, et al. Transforming growth factor-beta1 promoter polymorphism C-509T is associated with asthma. *Am J Respir Crit Care Med* 2004;169:214–219.
27. Shah R, Hurley CK, Posch PE. A molecular mechanism for the differential regulation of TGF-beta-1 expression due to the common SNP-509C-T (c.-1347C-T). *Hum Genet* 2006;120:461–469.
28. Suthanthiran M, Li B, Song JO, Ding R, et al. Transforming growth factor-beta-1 hyperexpression in African-American hypertensives: a novel mediator of hypertension and/or target organ damage. *Proc Nat Acad Sci* 2000;97:3479–3484.
29. Grutters JC, du Bois RM. Genetics of fibrosing lung diseases. *Euro Respir J* 2005;25: 915–927.
30. Celedon JC, Lange C, Raby BA, Litonjua AA, et al. The transforming growth factor-beta1 (TGFB1) gene is associated with chronic obstructive pulmonary disease (COPD). *Hum Mol Genet* 2004;13:1649–1656.
31. Vockley J, Rinaldo P, Bennett MJ, Matern D, et al. Synergistic heterozygosity: disease resulting from multiple partial defects in one or more metabolic pathways. *Mol Genet Metab* 2000;71:10–18.
32. Schuler AM, Gower BA, Matern D, Rinaldo P, et al. Synergistic heterozygosity in mice with inherited enzyme deficiencies of mitochondrial fatty acid β oxidation. *Mol Genet Metab* 2005;85:7–11.
33. Loyd JE, Primm RK, Newman JH. Familial primary pulmonary hypertension: clinical patterns. *Am Rev Respir Dis* 1984;129:194–197.
34. Simonneau G, Galie N, Rubin LJ, Langleben D, et al. Clinical classification of pulmonary hypertension. *J Am Coll Cardiol* 2004;43(12 suppl S):5S–12S.
35. Habashi JP, Judge DP, Holm TM, Cohn RD, et al. Losartan an AT1 antagonist, prevents aortic aneurysm in a mouse model of Marfan syndrome. *Science* 2006;12:117–121.
36. Weir BS. Genetic data analysis 2: methods for discrete population genetic data. Sunderland, MA: Sinauer Associates, Inc., 1996.
37. Nielsen D, Ehm MG, Weir BS. Detecting marker-disease association by testing for Hardy-Weinberg disequilibrium at a marker locus. *Am J Hum Genet* 1999;63:1531–1540.
38. Akhurst RJ. TGF beta signaling in health and disease. *Nat Genet* 2004;36:1024–1031.
39. Loeys BL, Chen J, Neptune ER, Judge DP, et al. A syndrome of altered cardiovascular, craniofacial, neurocognitive and skeletal development caused by mutations in TGFBR1 or TGFBR2. *Nat Genet* 2005;37:275–281.
40. Loeys BL, Schwarze U, Holm T, Callewaert BL, et al. Aneurysm syndromes caused by mutations in the TGF-beta receptor. *N Engl J Med* 2006;355:788–798.
41. Ghofrani HA, Weeger W, Grimminger F. Imatinib for the treatment of pulmonary arterial hypertension. *N Engl J Med* 2005;353:1412–1413.
42. Rondelet B, Kerbaul F, Van Beneden R, Hubloue I, et al. Prevention of pulmonary vascular remodeling and of decreased BMPR-2 expression by Losartan therapy in shunt-induced pulmonary hypertension. *Am J Physiol Heart Circ Physiol* 2005;289:H2319–H2324.
43. Robinson PN, Arteaga-Solis E, Baldock C, Collod-Beroud G, et al. The molecular genetics of Marfan syndrome and related disorders. *J Med Genet* 2006;10:769–787.
44. Lacro RV, Dietz HC, Wruck LM, Bradley TJ, et al. Rationale and design of a randomized clinical trial of β-blocker therapy (atenolol) versus angiotensin II receptor blocker therapy (losartan) in individuals with Marfan syndrome. *Am Heart J* 2007;154:624–631.