2012

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TGFβRIIb Mutations Trigger Aortic Aneurysm Pathogenesis by Altering Transforming Growth Factor β2 Signal Transduction

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Background—Thoracic aortic aneurysm (TAA) is a common progressive disorder involving gradual dilation of the ascending and/or descending thoracic aorta that eventually leads to dissection or rupture. Nonsydromic TAA can occur as a genetically triggered, familial disorder that is usually transmitted in a monogenic autosomal dominant fashion and is known as familial TAA. Genetic analyses of families affected with TAA have identified several chromosomal loci, and further mapping of familial TAA genes has highlighted disease-causing mutations in at least 4 genes: myosin heavy chain 11 (*MYH11)*, α-smooth muscle actin (*ACTA2)*, and transforming growth factor β receptors I and II (*TGFβRI* and *TGFβRII*).

Methods and Results—We evaluated 100 probands to determine the mutation frequency in *MYH11*, *ACTA2*, *TGFβRI*, and *TGFβRII* in an unbiased population of individuals with genetically mediated TAA. In this study, 9% of patients had a mutation in one of the genes analyzed, 3% of patients had mutations in *ACTA2*, 3% in *MYH11*, 1% in *TGFβRII,* and no mutations were found in *TGFβRI.* Additionally, we identified mutations in a 75 base pair alternatively spliced *TGFβRII* exon, exon 1a that produces the TGFβRIIb isoform and accounted for 2% of patients with mutations. Our in vitro analyses indicate that the *TGFβRIIb* activating mutations alter receptor function on TGFβ2 signaling.

Conclusions—We propose that TGFβRIIb expression is a regulatory mechanism for TGFβ2 signal transduction. Dysregulation of the TGFβ2 signaling pathway, as a consequence of *TGFβRIIb* mutations, results in aortic aneurysm pathogenesis. **(*Circ Cardiovasc Genet*. 2012;5:621-629.)**

Key Words: aneurysm ■ aorta ■ cardiovascular diseases ■ genetics ■ transforming growth factor-β pathway

Thoracic aortic aneurysm (TAA) is a common progressive disorder involving gradual dilation of the ascending and descending thoracic aorta that eventually leads to dissection or rupture. TAAs are often clinically silent and unsuspected until dissection or rupture occurs. The result is significant morbidity and mortality despite advances in surgical and percutaneous treatments for aortic disease. Although TAA is often a feature of Mendelian complex connective tissue disorders such as Marfan syndrome, Ehlers-Danlos syndrome type IV, or Loeys-Dietz syndrome (LDS), most TAAs occur as isolated nonsyndromic disorders. Nonsydromic TAAs can also be a familial disorder that is usually transmitted in a monogenic autosomal dominant fashion. These genetically triggered TAAs account for ≈20% of TAAs.[1–6] Genetic analyses of familial TAA (FTAA) have identified several chromosomal loci. Further mapping of FTAA genes has highlighted disease-causing mutations in at least 4 genes: myosin heavy chain 11 (*MYH11)*, α-smooth muscle actin (*ACTA2)*, and transforming growth factor β receptors I and II (*TGFβRI* and *TGFβRII*).[7–10] Recent studies have also identified mutations in 2 novel genes, *MYLK* and *SMAD3*, that are linked to syndromic aortic aneurysms and dissections.[11,12] Also, disease genes remain to be determined at additional loci such as *AAT1* (also known as *FAA1*) on chromosome 11q23[13] and *AAT2* (also known as *TAAD1*) on chromosome 5q13.[14]

Clinical Perspective on p 629

Because of the identification of *TGFβRI* and *TGFβRII* mutations in aortic aneurysm syndromes such as LDS, considerable attention has been devoted to the role that TGFβ may play in FTAA pathogenesis. The TGFβ receptor superfamily is composed of cytokines that control numerous diverse cellular processes including cell proliferation, differentiation, angiogenesis, and modification of the extracellular matrix.[13–16] Canonical TGFβ signaling is initiated when a TGFβ ligand binds to TGFβRII, resulting in the recruitment of TGFβRI. On ligand binding, TGFβRII activates TGFβRI via transphosphorylation of its kinase domain and propagates downstream signaling actions. Receptor-regulated (R-) Smads are substrates of the TGFβRI kinase, and cytoplasmic phosphorylation of R-Smads allows for translocation of the Smad complexes to the nucleus to regulate transcription of target genes.[17]

Received July 22, 2011; accepted October 5, 2012.

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Previous studies identified mutations in *TGFβRI, TGFβRII, ACTA2*, and *MYH11* in individuals with FTAA. In most cases, genetic screenings for mutations in these genes have focused primarily on patients referred to genetic subspecialists with either an extensive family history or obvious features of a complex Mendelian connective tissue disorder, and, therefore, these patients have an increased likelihood of harboring a mutation. However, such individuals represent a small subset of those with genetically mediated TAA. The vast majority of patients present with limited or unknown family history and are without evidence of a complex syndromic disorder. These patients represent diagnostic dilemmas for practicing physicians. This study addresses the potential impact of genetic testing for these 4 TAA genes on clinical management of patients with TAA. We determined the frequency of mutations in these 4 TAA genes in an unbiased population that is more representative of the population of individuals with genetically mediated TAA seen in cardiovascular clinical practice.

Methods

Patient Cohort Collection

The cohort of patients enrolled in this study consisted of 100 consecutive adult probands from a clinical population with nonsyndromic, potentially genetically triggered aortic aneurysms. Patients with FTAA were collected from those presenting to cardiologists and cardiothoracic surgeons at Weill Cornell Medical Center. Written informed consent was obtained from all subjects according to a protocol approved by the institutional review board of Weill Cornell Medical College. To enroll, subjects needed to have been diagnosed with thoracic aortic dilation, aneurysm, or dissection and meet at least 1 of these criteria:

1. Age at diagnosis of aortic disease <50 years.
2. Positive family history of aortic aneurysm or dissection in at least 1 first- or second-degree relative.
3. Features of a connective tissue disorder, such as arachnodactyly, pectus carinatum, or pectus excavatum.

These inclusion criteria were established to represent patients who might reasonably be clinically suspected to have a genetically mediated disorder. Patients were excluded if they met clinical diagnostic criteria for Marfan syndrome, LDS, or Ehlers-Danlos syndrome because pathogenesis for these rare syndromes are well known and do not generally present diagnostic dilemmas to physicians.

DNA Isolation and Mutation Analysis

Blood or saliva samples were obtained from patients. Genomic DNA was isolated from lymphoblasts separated from whole blood (QIAamp DNA Blood kit, Qiagen, Valencia, CA) and saliva (Oragene-DNA kit, DNA Genotek, Kanata, Ontario, Canada) per manufacturer's instructions. Exons of *ACTA2*, *MYH11*, *TGFβRI*, and *TGFβRII* were polymerase chain reaction (PCR) amplified with gene-specific primers from genomic DNA isolated from each patient. Primer sequences are available on request. Additional mutational analyses of *TGFβRII* focused on an alternatively spliced exon, exon 1a that substitutes a 26 amino acid peptide for Val51 in the receptor's extracellular domain. This resultant TGFβRII is often referred to as TGFβRIIb, and the specific properties and function of TGFβRIIb are not well documented.[18]

PCR products were purified by vacuum filtration using a MultiScreen-PCR filter plate (Millipore, Darmstadt, Germany) per manufacturer's instructions. Purified PCR products were bidirectionally sequenced and analyzed on an automated sequencer (ABI 3130XL) with BigDye Terminator v3.1 (Applied Biosystems, Foster City, CA). Exons with sequence variants were analyzed in family members when available. In addition, a minimum of 200 control chromosomes from a population of normal samples (mixed-ethnicity unaffected individuals without known aortic disease) were also analyzed either by restriction fragment length polymorphism analysis or denaturing high-performance liquid chromatography on a WAVE Nucleic Acid Fragment Analysis System (Transgenomic, Omaha, NE). Additional sets of ethnically matched controls were used as noted in the results section below.

Sequence variants were considered mutations if they (1) caused a nonsynonymous amino acid change or could potentially alter protein structure, (2) were absent from a population of at least 200 ethnically matched control chromosomes, and (3) co-segregated with disease in the family if family member samples were also available for analysis. In addition, we examined the National Center for Biotechnology Information (NCBI) single-nucleotide polymorphism (SNP) database of 4356 chromosomes for *ACTA2*, *MYH11*, *TGFβRI,* and *TGFβRII* polymorphisms in genome build 37.3 released in October 2011.

RNA Analyses

Total RNA was isolated from either lymphocytes or homogenized human aortic tissue using TRIzol reagent (Invitrogen, Grand Island, NY) per manufacturer's instructions. RNA was subjected to reverse transcriptase PCR (RT-PCR; One-Step RT-PCR Kit, Qiagen) to preferentially amplify either the *TGFβRII* or *TGFβRIIb* isoform with exon-specific primers surrounding, or internal to, the alternatively spliced exon. RT-PCR reactions were performed under the following conditions: 50°C for 30 minutes (cDNA synthesis), 95°C for 15 minutes (polymerase heat activation), followed by 94°C for 45 seconds, 52°C for 80 seconds, and 72°C for 60 seconds for 35 cycles.

Plasmid Constructs

Full-length cDNAs of both *TGFβRII* and *TGFβRIIb* were reverse transcribed as described above from RNA extracted from patient fibroblasts using primers immediately flanking the coding region of *TGFβRII*. RT-PCR products were cloned into the pCR2.1-TOPO vector using the TOPO TA cloning kit (Invitrogen). Plasmid DNA was isolated (QIAprep Miniprep kit, Qiagen, La Jolla, CA). The entire coding region of *TGFβRII* and *TGFβRIIb* in each construct was sequenced bidirectionally in each cDNA construct to confirm the correct full-length sequence for both. The H56N *TGFβRIIb* mutation was generated by site-directed mutagenesis (QuikChange Kit, Stratagene, La Jolla CA) per manufacturer's instructions. The D40N *TGFβRIIb* mutation was generated by Overlap Extension PCR.[19] Initial PCR reactions were performed under the following conditions: 95°C for 1 minute, 50°C for 2 minutes, and 72°C for 2 minutes for 25 cycles. PCR products were subjected to gel electrophoresis and gel purified (QIAquick Gel Extraction Kit, Qiagen) per manufacturer's instructions. Subsequent PCR reactions were performed using purified DNA from the initial PCR product using the same cycle conditions.

Wild-type TGFβRII as well as wild-type and mutant isoforms of TGFβRIIb were subcloned into *Xho*I and *BamH*I sites of the pcDNA3.1(-) expression vector 3′ to a cassette encoding a Kozak sequence to generate TGFβRII- or TGFβRIIb-cDNA3.1. PCR amplification with *Xho*I-Kozak-TGFβII-F and *BamH*I-TGFβRII-R primers facilitated cloning into pcDNA3.1 (Invitrogen). The entire coding region of each *TGFβRII* and *TGFβRIIb* construct was bidirectionally sequenced to confirm the correct full-length sequence.

Cell Culture, Transfection, and TGFβ Stimulation

A skin biopsy containing primary dermal fibroblasts from an individual harboring the H56N *TGFβRII* mutation was cultured in DMEM (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum and 0.1 mg/mL primocin. Cells were maintained and studied at low passage (passages 2–5). Normal human dermal fibroblasts and L6 rat myoblast cells were obtained from American Type Culture Collection and grown in DMEM with 10% fetal bovine serum.

Low-passage primary dermal fibroblasts (passages 2–5) were serum starved for 24 hours and then stimulated with 5 ng/mL recombinant human TGFβ1 or recombinant human TGFβ2 (R&D Systems, Minneapolis, MN) in the presence of 10% serum. After stimulation, cells were washed twice in cold PBS containing 1 mmol/L Na_3VO_4 and lysed at 0, 0.5, 1, 2, 4, and 24 hours poststimulation in radioimmunoprecipitation (RIPA) buffer (50 mmol/L Tris HCl pH7.6,

150 mmol/L NaCl, 1% IGEPAL, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors (1X Protease Inhibitor Cocktail Tablets, Roche, Indianapolis, IN; 1% Ser/Thr Protein Phosphatase Inhibitor Cocktail, Millipore, Billerica, MA) for subsequent Western blot analysis as described below. Cell stimulation was performed in triplicate.

One day before transfection, 3×10^5 L6 cells were plated and transfected with 500 ng of either wild-type (wt) or mutant TGFβRII-cDNA3.1 constructs (wt-TGFβRII-, wt-TGFβRIIb-, H56N-TGFβRIIb-, or D40N-TGFβRIIb-cDNA3.1) in low serum media using Lipofect AMINE (Invitrogen). Cells either remained unstimulated or were stimulated with 50 pmol/L TGFβ1 or 50 pmol/L TGFβ2 for 0, 0.5, 1, or 2 hours and lysed as described above in RIPA buffer supplemented with protease inhibitors for Western blot analysis. All cell transfections and stimulations were performed in triplicate.

SDS-PAGE and Western Blotting

Lysate protein concentrations were determined (CoomassiePlus Bradford Assay kit, Pierce, Rockford, IL). Twenty micrograms of cell lysate were electrophoresed on 10% polyacrylamide gels (Pierce) and transferred to polyvinylidene fluoride membrane (GE Healthcare, Waukesha, WI). Protein expression was determined by Western blotting with primary antibodies to anti-phosphorylated Smad2 (pSMAD2, Cell Signaling, Danvers, MA) or anti-β-actin. (Sigma, St. Louis, MO). Bound antibodies were detected by incubation with goat anti-rabbit secondary antibody (Cell Signaling) followed by chemiluminescence (ECL Plus, GE Healthcare). Densitometry was performed on a BioRad Gel Doc MultiAnalyst system.

Statistical Analysis

All values are expressed as mean±SEM or percentage and 95% confidence intervals for categorical variables. Statistical analyses were performed using ANOVA and Student *t* test. $P<0.05$ was considered significant. A Sharp-Wilk normality test of our Western blot data revealed that it followed normal distributions with a $P\geq0.01$ and had equal variance. Binomial power calculations indicate that our power to detect mutations with a prevalence as low as 1% is 55% in a sample of 200 control chromosomes and 95% in 588 control chromosomes. Also, binomial power calculations indicate that our power to detect NCBI SNPs with a prevalence of 5/4356 chromosomes is 21% in 200 chromosomes and 49% in 588 chromosomes, whereas our detection power is 37% in 200 chromosomes and 74% in 588 chromosomes with an SNP having a prevalence of 10/4356 chromosomes.

Table. Description of Cohort

Sex, n	
Male	77
Female	23
Ethnicity, n	
White	80
White/Hispanic	6
White/Native American	2
Black	2
Black/Hispanic	2
Asian	2
Other	6
Age at enrollment, y	
Mean	53
Median	52
Range	21–93
Body surface area (BSA), m^2	
Mean (n=65)	1.99
Median	2.00
Aortic surgery, n	42
Dissection, n	27
Inclusion criteria, n	
Diagnoses <50 y old	64
Connective tissue abnormality	14
Family history	67

Results

Patient Cohort

The cohort consisted of predominantly male patients (77 men and 23 women) (Table). This distribution is consistent with previous studies in which men predominate in a series of clinically apparent genetically meditated TAAs.[1] The majority of patients were white (80/100), and the remainder were of Hispanic, black, Native American, or Asian descent. Patients ranged in age from 21 to 93 years of age with an average age of 53. The average weight of patients was 85.1 kg, and the average height was 177.3 cm with an average body surface area of 2 m^2. Of the 100 patients, 64 were diagnosed at <50 years of age. Sixty-seven had a family history of TAA or thoracic aortic dissection. Only 14 exhibited connective tissue abnormalities, including joint hypermobility, pectus excavatum, and pectus carinatum, but none met or nearly met diagnostic criteria for Marfan or other syndromes.[20] At the time of enrollment, 42 patients had previously undergone aortic surgery. Aortic dissections had been reported in 27 of the patients.

Aneurysm Gene Mutational Analyses

All patients in this cohort underwent sequencing-based mutational analyses of the *ACTA2*, *MYH11*, *TGFβRI,* and *TGFβRII* genes. Sequencing of the *ACTA2* gene revealed 3 missense mutations: T108M, R118Q, and G270E (Figure 1). Two intronic polymorphisms were seen; however, no exonic polymorphisms were detected. The R118Q mutation in family JNW has been previously reported in 2 other TAA families.[21] T108M and G270E were not found in 200 white/Northern European control chromosomes. All 3 affected probands had TAAs, 2 of which led to acute dissections, and all required surgery. Both the R118Q (family JNW) and G270E (family ANS) mutations co-segregated with disease in families. Family members of SY92, who carried the T108M mutation, were unavailable for genetic analysis. Patient SY92 also had an atrial septal defect and patent ductus arteriosus (PDA). None of the *ACTA2* mutations was found in the NCBI SNP database.

Three mutations and 13 polymorphisms were identified in the *MYH11* gene (Figure 2), including 2 missense mutations (R1590Q and E1899D) and 1 splice site alteration: a 7 nucleotide substitution located 5 base pairs 3′ to exon 27. None of the variants was detected in 200 white/Northern European control chromosomes. The mutations co-segregated with aortic disease in families JNE and ANHH. Family members of patient ANO II-2 (carrying a 7 nucleotide substitution located 5 base pairs 3′ to exon 27) were unavailable for genetic analysis. Individual ANHH II-2 exhibited TAAs and also a bicuspid aortic valve; however, ANO II-2 had a tricuspid aortic valve. Individual JNE II-1 exhibited TAA, resulting in 2 dissections. None of these mutations was identified in the NCBI SNP database except for E1899D *MYH11* that was found in 10/4356

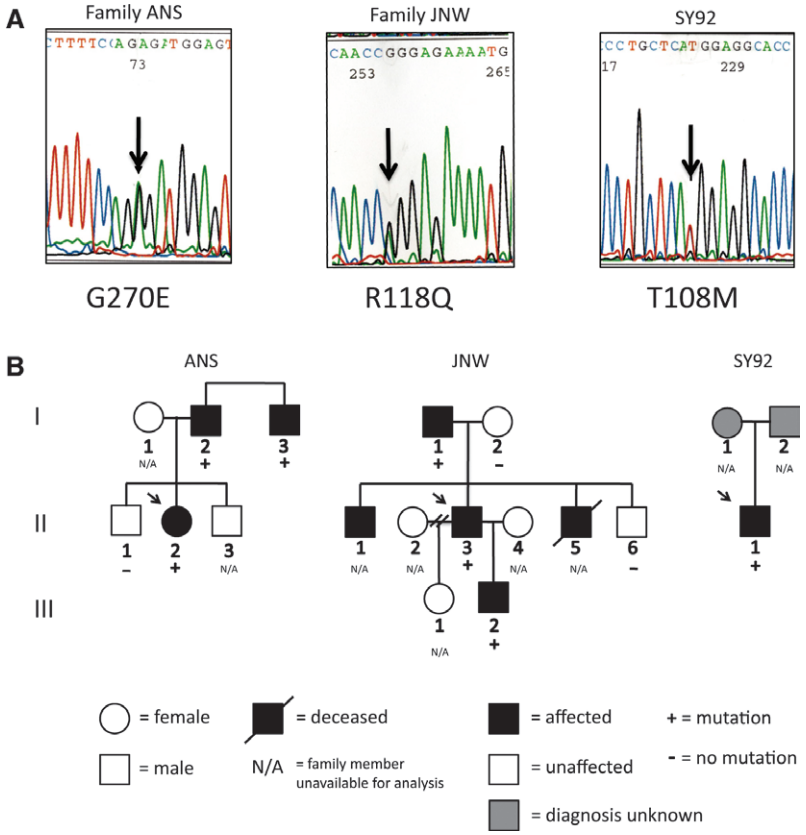

Figure 1. Mutational analysis of *ACTA2* in families ANS, JNW, and SY92. **A**, Automated sequence analyses of *ACTA2* from affected individuals in families ANS, JNW, and SY92 are shown. A 1-bp deletion in families ANS, JNW, and SY92 (arrow) generates a missense mutation indicated by the amino acid code. **B**, Pedigrees of families ANS, JNW, and SY92 with an *ACTA2* mutation indicate that mutations co-segregate with disease. Mutations are present only in family members affected with thoracic aortic aneurysm. Arrow indicates family proband. N/A indicates not applicable.

chromosomes corresponding to a frequency of 0.23%. The significance of this polymorphism is unknown given that the NCBI database includes patients with cardiovascular disease whose aortic aneurysm status is unknown. In our study, we screened 200 chromosomes from control patients who were unaffected individuals without known aortic disease.

Members of the cohort were also screened for mutations in both *TGFβR* type I and type II genes, *TGFβRI* and

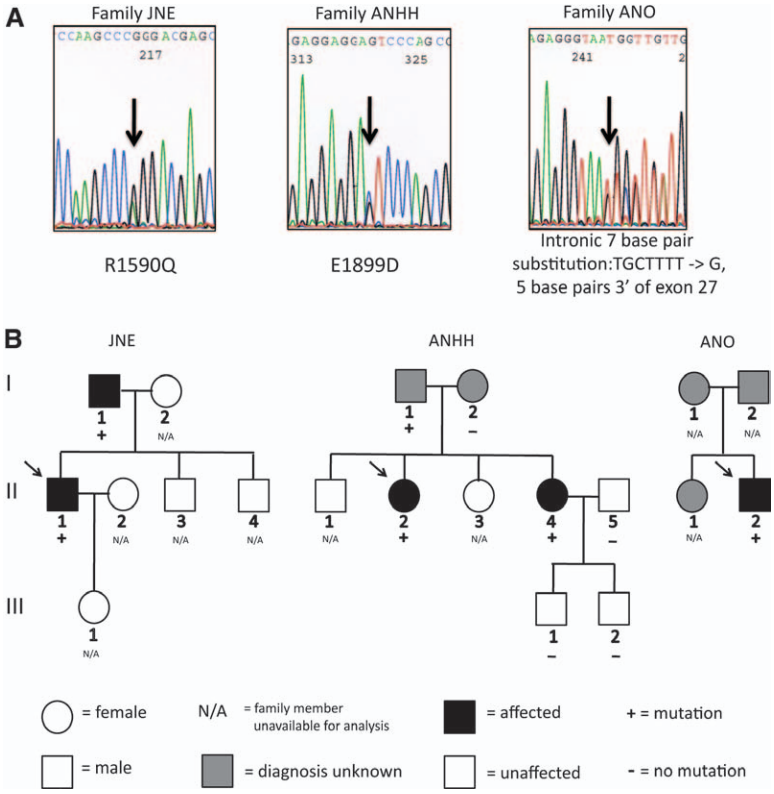

Figure 2. Mutational analysis of myosin heavy chain 11 (*MYH11*) in families JNE, ANHH, and ANO. **A**, Automated sequence analyses of *MYH11* from affected individuals in families JNE, ANHH, and ANO are shown. A 1-bp deletion in families JNE and ANHH (arrow) generates a missense mutation indicated by the amino acid code. A 7-bp intronic substitution in family ANO (arrow) produces a splice site alteration located at the nucleotide position relative to the preceding exon. **B**, Pedigrees of families JNE, ANHH, and ANO are shown and indicate that *MYH11* mutations co-segregate with disease. Mutations are present only in family members affected with thoracic aortic aneurysm. Arrow indicates family proband.

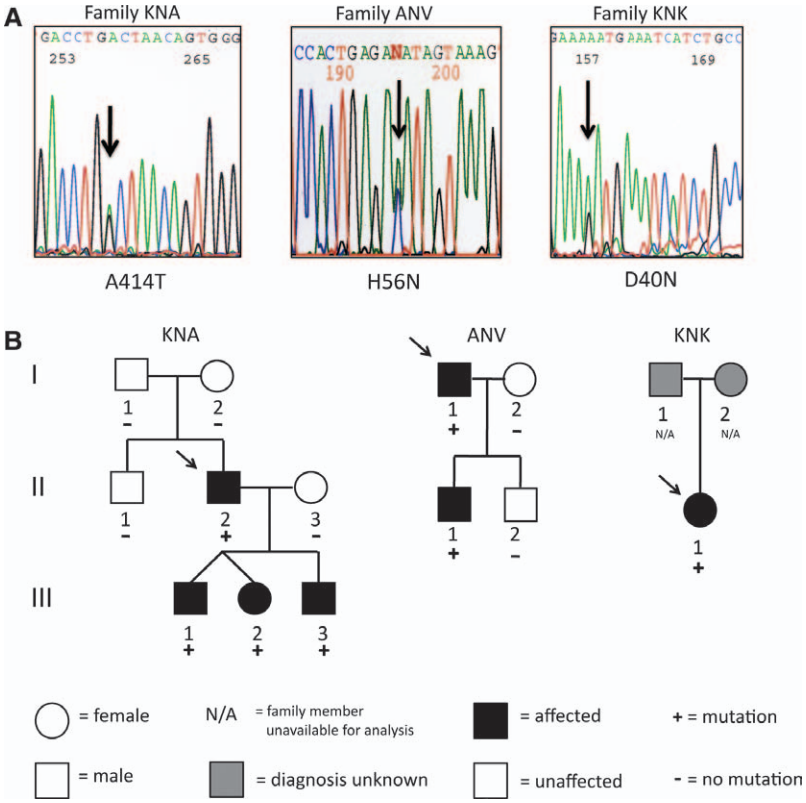

Figure 3. Transforming growth factor *TGFβRII* and *TGFβRIIb* mutations in families KNA, ANV, and KNK. **A**, Automated sequence analyses of *TGFβRII* and *TGFβRIIb* from affected individuals in families KNA, ANV, and KNK are shown. A 1-bp deletion in families KNA, ANV, and KNK (arrow) generates a missense mutation indicated by the amino acid code. **B**, Pedigree of family KNA with a *TGFβRII* mutation and families ANV and KNK with *TGFβRIIb* mutations indicate that mutations co-segregate with disease. Mutations are present only in family members affected with thoracic aortic aneurysm. Arrow indicates family proband.

TGFβRII. In *TGFβRI*, 3 exonic polymorphisms and no mutations were found. Two exonic polymorphisms and 1 mutation were detected in *TGFβRII* (Figure 3). The *TGFβRII* missense mutation (A414T) in patient KNA II-2 is located in the protein's kinase domain, co-segregates with disease in the family, and was absent in 206 ethnically matched (Ashkenazi Jewish) control chromosomes. All 4 family members carrying the mutation were diagnosed with aortic aneurysm. Three of the 4 also have pectus excavatum, one of whom was also diagnosed with an ostium secundum atrial septal defect. There was no evidence of other LDS features such as vascular tortuosity or bifid uvula in probands or family members. This *TGFβRII* mutation was not found in the NCBI SNP database.

TGFβRII has an additional alternatively spliced isoform containing a 75 base pair exon (exon 1a) in its extracellular domain that produces TGFβRIIb. *TGFβRII* exon 1a was also sequenced in all 100 individuals. No polymorphisms were found in exon 1a, but 2 missense mutations were identified. The D40N mutation (family KNK) was not detected in 224 white/Northern European control chromosomes. The H56N mutation (family ANV) co-segregated with disease in the family and was absent from 588 control chromosomes including 384 ethnically matched (Ashkenazi Jewish) chromosomes. KNK II-1 had a TAA and also a bicuspid aortic valve. ANV II-1 had an aortic aneurysm and pectus carinatum. H56N was not found in the NCBI SNP database, but D40N *TGFβRIIb,* identified in patient KNKII-1 for which we were unable to determine co-segregation with disease, was found in 5/4356 chromosomes corresponding to a frequency of 0.11%. The significance of this polymorphism is unknown given that the NCBI database includes patients with cardiovascular disease whose aortic aneurysm status is unknown. In our study, we screened 224 chromosomes from control patients who were unaffected individuals without known aortic disease.

In total, 9 of 100 probands were found to have a mutation in 1 of the 4 genes analyzed. No genotype-phenotype correlations were observed among the probands' available family members, and mutations within the same gene did not necessarily correspond to any specific phenotypic variation. Families ANV, KNK, and KNA each have *TGFβRII* mutations yet exhibit considerable interfamilial variation. Four of the 7 individuals with a *TGFβRII* mutation (including those with a mutation in the alternatively spliced exon) exhibited noncardiovascular connective tissue abnormality. Two were diagnosed with a congenital heart defect.

Although *MYH11* mutations have been previously associated with PDA,[9] none of the individuals with *MYH11* mutations detected in the current study was known to have PDA. Livedo reticularis or iris flocculi were not observed in individuals in this study with *ACTA2* mutations.

TGFβRII Expression and Activity

Previous studies[22] established that TGFβR kinase domain mutations inactivate the receptor, although downstream TGFβ signaling is paradoxically increased. Similarly, we identified the A414T *TGFβRII* mutation in a patient and examined its kinase activity through in vitro expression of A414T-TGFβRII in a luciferase vector. Mutant TGFβRII was inactive (data not shown). In this study, we sought to understand how the novel *TGFβRIIb* mutations we observed outside of the TGFβRII kinase domain might alter TGFβ signaling. Tissue expression patterns of TGFβRII alternative splicing have not been

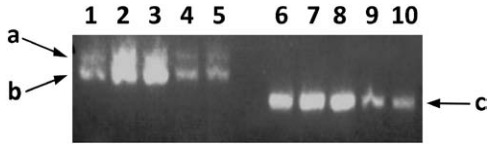

Figure 4. mRNA expression of *TGFβRII* isoforms in aortic tissue and lymphocytes. RT-PCR performed using various pairs of *TGFβRII* and *TGFβRIIb* isoform specific primers to amplify exons 1, 1a, 2, and 3 (a), exons 1, 2, and 3 (b), and exons 1a, 2, and 3 (c) from isolated human aortic tissue (lanes 1–2) and lymphocyte (lanes 3–4) RNA.

established. Using RT-PCR, we determined that both spliced isoforms, TGFβRII and TGFβRIIb, are expressed in the human aortic wall and lymphocytes (Figure 4) and also in cultured dermal fibroblasts and aortic smooth muscle cells (not shown).

To determine the effects of mutant TGFβRIIb on TGFβ signaling, we compared relative levels of TGFβ1 and TGFβ2 signaling (indicated by pSMAD2 levels) in normal dermal fibroblasts with dermal fibroblasts isolated from individual ANV I-1 who is heterozygous for the H56N *TGFβRIIb* mutation (Figure 5). Although the specific contributions of canonical TGFβ signaling via Smads versus noncanonical TGFβ signaling via extracellular signal-regulated kinase mitogen-activated protein kinase (MEK/ERK) pathways to the pathogenesis of specific aneurysm and aneurysm related phenotypes remain under active investigation,[23–25] pSMAD2 provides a valuable biomarker of TGFβ activity.[26] On TGFβ1 stimulation of normal dermal fibroblasts, we observed an increase in pSMAD2 levels peaking at 0.5 hours poststimulation before declining by 4 hours. By contrast, TGFβ1 stimulation of ANV I-1 dermal fibroblasts resulted in delayed SMAD2 phosphorylation that peaked at 2 hours. On stimulation with TGFβ2, normal dermal fibroblasts exhibited similar

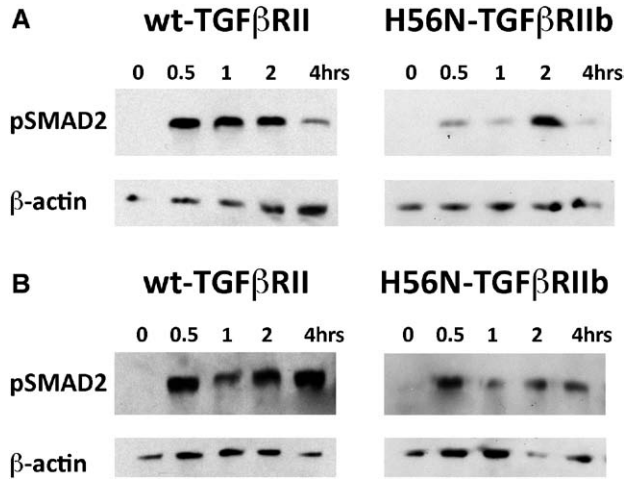

Figure 5. TGFβ1 and TGFβ2 signaling in dermal fibroblasts. **A** and **B**, Cultured fibroblasts were isolated from normal patient with wild type (wt)-TGFβRII and ANVI-1 patient with H56N-TGFβRIIb mutation. Representative Western blot analyses shown for pSMAD2 protein expression in wt-TGFβRII or H56N-TGFβRIIb fibroblasts stimulated with either TGFβ1 (**A**) or TGFβ2 (**B**) for indicated time points. Corresponding β-actin blots shown. **A**, H56N-TGFβRIIb dermal fibroblasts exhibit delayed TGFβ1 signaling compared with normal dermal fibroblasts. **B**, H56N-TGFβRIIb dermal fibroblasts exhibit decreased TGFβ2 signaling compared with normal dermal fibroblasts.

kinetics to TGFβ1 stimulation; pSMAD2 levels peaked at 0.5 hours, although high levels of pSMAD2 persisted even 4 hours poststimulation. TGFβ2 stimulation of ANV I-1 dermal fibroblasts exhibited distinct kinetics of SMAD2 phosphorylation. Although pSMAD2 levels peaked at 0.5 hours, these levels rapidly declined by 1 hour and were markedly reduced at all time points compared with normal dermal fibroblasts.

Because dermal fibroblasts from individual ANV I-1 express both wt- and mutant TGFβRIIb isoforms, one cannot distinguish the activities of each isoform in these cells. Therefore, we utilized L6 rat myoblast cells lacking both endogenous TGFβRIIb and TGFβRIII to compare TGFβ signaling activities in wt- and mutant TGFβRIIb. L6 myoblasts were transfected with constructs encoding wt-TGFβRII, wt-TGFβRIIb, H56N-TGFβRIIb, or D40N-TGFβRIIb and then stimulated with 50 pmol/L TGFβ1 or TGFβ2. Our preliminary examination of pSMAD2 levels measured during the first 2 hours poststimulation showed that TGFβRII activity peaked at 0.5 hours in TGFβ1-stimulated L6 cells, whereas it peaked at 1 hour in TGFβ2-stimulated cells. Therefore, in subsequent studies, we assessed peak TGFβ receptor activity in genetically engineered L6 cells after stimulation with either TGFβ1 for 0.5 hours (Figure 6) or TGFβ2 for 1 hour (Figure 7). L6 cells transfected with either wt-TGFβRII or wt-TGFβRIIb exhibited comparable responsiveness to TGFβ1 (Figure 6A, n=3). Similarly, introduction of neither H56N-TGFβRIIb nor D40N-TGFβRIIb significantly modified TGFβ1 responsiveness (Figure 6B, n=3). However, when cells were stimulated with TGFβ2 (Figure 7), pSmad2 levels were reduced by 74% in cells transfected with wt-TGFβRIIb compared with those transfected with wt-TGFβRII (Figure 7A; n=3, P=0.02). Introduction of either H56N-TGFβRIIb or D40N-TGFβRIIb, then, ablated this reduction in receptor activity, and both resulted in a nearly 3-fold increase in pSmad2 levels (Figure 7B; n=3, P=0.009 and P=0.02, respectively).

Discussion

FTAA is a clinically heterogeneous disorder exhibiting variation in both age of onset and degree of aortic dilatation prior to dissection. FTAA can be part of a complex syndrome, such as LDS, or an isolated finding. The 4 genes analyzed in this study (*ACTA2*, *MYH11, TGFβRI*, and *TGFβRII*) were initially identified as associated with syndromic FTAA, and the cause of FTAA in many families remains unknown. The utility of mutational analyses in clinical strategies for an isolated FTAA diagnostic workup is unclear.

The principal goal of our study was to address the potential value of clinical genetic testing of *ACTA2*, *MYH11, TGFβRI*, and *TGFβRII* in nonsyndromic FTAA to improve patient care and diagnosis. Although these 4 FTAA-causative genes are known to be prevalent in cohorts ascertained for molecular genetic studies, their contribution to disease in a population relevant to clinical practice has not previously been studied. In this study, we determined the frequency of mutations in these 4 TAA genes in a cohort routinely seen in cardiology clinical practice. Individuals diagnosed with known Marfan syndrome, LDS, or Ehlers-Danlos syndrome were excluded. In this study, 9% of patients had a mutation in 1 of the genes analyzed. Three percent of patients had mutations in *ACTA2*, 3% in *MYH11*, and 3% in *TGFβRII*. No mutations were found

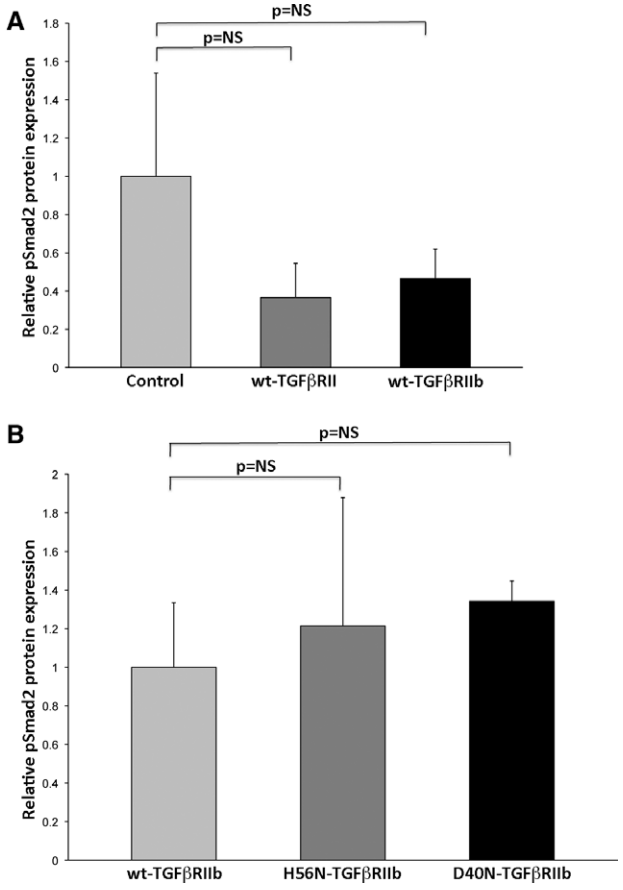

Figure 6. In vitro TGFβ1 stimulation does not alter TGFβRII and TGFβRIIb activity. **A**, Relative quantification of pSmad2 expression after in vitro TGFβ1 stimulation. Bar graph displays densitometric quantification of pSmad2 protein expression relative to β-actin in untransfected control, wild-type (wt)-TGFβRII and wt-TGFβRIIb L6 cells (n=3). No significant change in TGFβRII or TGFβRIIb activity compared with control cells. **B**, Relative quantification of pSmad2 expression after in vitro TGFβ1 stimulation. Bar graph displays densitometric quantification of pSmad2 relative to β-actin in control wt-TGFβRIIb, H56N-TGFβRIIb, and D40N-TGFβRIIb L6 cells (n=3). No significant change in activity in mutant TGFβRIIb cells compared with wt-TGFβRIIb cells in response to 0.5 hour 50 pmol/L TGFβ1 stimulation. Values normalized to control L6 cells. All data presented as mean±SEM. *P* values shown. NS indicates not significant.

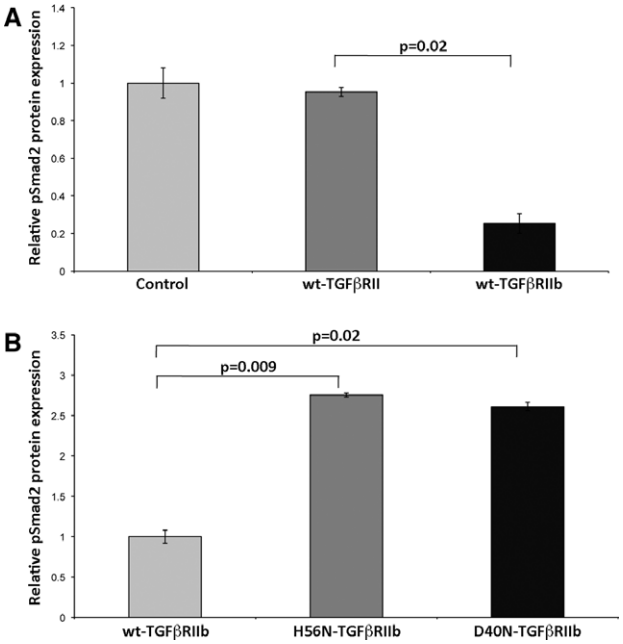

Figure 7. In vitro TGFβ2 stimulation alters TGFβR2 and TGFβRIIb activity. **A**, Relative quantification of pSmad2 expression after in vitro TGFβ2 stimulation. Bar graph displays densitometric quantification of pSmad2 protein expression relative to β-actin in untransfected control, wild-type (wt)-TGFβRII and wt-TGFβRIIb L6 cells (n=3). wt-TGFβRIIb cells exhibit decreased TGFβ2 signaling compared with wt-TGFβR2 cells. **B**, Relative quantification of pSmad2 expression after in vitro TGFβ2 stimulation. Bar graph displays densitometric quantification of pSmad2 relative to β-actin in control wt-TGFβRIIb, H56N-TGFβRIIb, and D40N-TGFβRIIb L6 cells (n=3). Mutant TGFβRIIb cells exhibit increased response after 1 hour 50 pmol/L TGFβ2 stimulation compared with wt-TGFβRIIb cells. Values normalized to control L6 cells. All data presented as mean±SEM. *P* values shown.

in *TGFβRI,* consistent with the reported rarity of *TGFβRI* mutations outside of LDS.[27–29]

Previous studies reported higher rates of mutation (14% in *ACTA2* and 5%–10% in *TGFβRII*) than observed here on screening the same genes.[9,10,30] Our study differs from those studies whose cohorts may have been ascertained through family-based programs and medical genetic clinics to which patients are largely referred if they are believed to have signs or symptoms of known disorders, such as LDS, Marfan syndrome, and Ehlers-Danlos syndrome. Patients in those studies are more likely to harbor a mutation in one of these genes, and our cohort may be more representative of the patient population routinely presenting to cardiovascular clinical practices.

Our study provides an estimate of the potential value of genetic testing for mutations in known aortic aneurysm disease genes as part of the diagnostic workup of these patients who are often seen by the general cardiologist or cardiothoracic surgeon. The 95% confidence interval for the point estimate of 9% in our population is consistent with finding a potentially causative mutation in 5% to 16% of such patients in cardiovascular clinical practices. Genetic testing can be a valuable adjunct for diagnostic management of aortic aneurysm because this disorder often goes undiagnosed until a dissection or rupture occurs. Individuals identified by genetic testing as at risk for aortic aneurysm development can undergo interval imaging earlier to monitor the progression of aortic dilation and to facilitate intervention prior to rupture and dissection. This study provides a foundation for future studies that will likely provide insight into how enhanced diagnostic algorithms incorporating routine TAA genetic testing can improve patient outcomes and survival. Regardless, our study critically highlights the need for further FTAA gene identification because most genetically triggered aortic aneurysm patients in our study had no evidence of mutation in any of the genes analyzed. With clinical deployment of exonic and genomewide sequencing that do not rely on family-based analyses, cohorts such as the one followed here will provide a rich source for such gene identification.

Although no genotype-phenotype correlation was found in this study, the statistical power to detect correlations may have been inadequate because of the small number of individuals with a mutation. Nonetheless, the study already highlights certain clinical diagnostic hazards. For instance, PDA

has been strongly associated with *MYH11* mutations in fact, although we observed PDA in the setting of *ACTA2* mutations as well. Thus, the presence of PDA should not provoke the presumption of *MYH11* mutations.

TGFβ signaling has become an emerging target for novel therapies for aortic aneurysms. Previous studies have established that dysregulated TGFβ signaling contributes to aortic aneurysms.[22,31] However, a paradox in the mode of pathogenesis obfuscates a clear functional role for TGFβ in aneurysm development.[16] Identification of *TGFβR* mutations in LDS and characterization of novel *TGFβRIIb* mutations in an alternatively spliced gene segment in our study highlight the contribution of enhanced TGFβ signaling to FTAA. Although previously identified *TGFβR* mutations modified the receptors' kinase domain, this study identifies novel mutations in an alternatively spliced segment of *TGFβRII* that is not involved in kinase activity. Functional analyses of several kinase domain mutations have revealed consequent loss-of-function that nevertheless displayed a paradoxical enhancement of TGFβ signaling in patient aortic tissue. By contrast, mutations in the alternatively spliced segment of *TGFβRIIb* described here are unique because they augment receptor activity, and these findings prompted us to evaluate the biochemical significance of these mutations.

Prior mutational analyses of *TGFβRII* have rarely included the alternatively spliced segment.[9,27] Little is known about the function of this alternative receptor isoform.[32,33] A previous study asserted that TGFβRII requires an accessory receptor, TGFβRIII, for efficient binding of TGFβ2 and subsequent signaling.[34] Rotzer et al[33] proposed that TGFβRIIb alone is capable of binding TGFβ1, TGFβ2, and TGFβ3 whereas del Re et al[32] suggested that TGFβRIIb alone is capable of binding only TGFβ1 and TGFβ3. In contrast to the binding data, del Re et al[32] further proposed that TGFβRIIb mediates in vitro TGFβ2 signaling in a dose-dependent manner. We demonstrate that the segment encoded by exon 1a does not alter receptor function on stimulation with TGFβ1, but does alter TGFβ2 signaling. TGFβRIIb has a lower TGFβ2-stimulated activity than TGFβRII, and mutations in this alternatively spliced segment reverse this effect, increasing receptor activity levels similar to that of prototypical TGFβRII. We then propose that TGFβRIIb expression is a regulatory mechanism for TGFβ2 signal transduction, and dysregulation of the TGFβ2 signaling pathway resulting from *TGFβRIIb* mutations can contribute to aneurysm pathogenesis.

Regulation of the TGFβ signaling pathway is important in determining cellular outcome, and the underlying mechanisms are complex. This pathway depends on several factors including the stoichiometric balance of TGFβ ligands and receptors expressed within the cell. Although TGFβRIIb binds TGFβ1,[32,33] we did not observe a change in TGFβI signaling in response to mutant TGFβRIIb expression. On TGFβ2 stimulation of cells expressing either wt-TGFβRII or wt-TGFβRIIb, we observed significantly less TGFβRIIb activity relative to TGFβRII activity. However, mutant TGFβRIIb isoforms ablated this reduction in receptor activity by increasing TGFβ2-stimulated TGFβRIIb activity to levels equivalent to that of wt-TGFβRII. The increase in TGFβ2 signaling that we observe may be related to complex stoichiometric interactions at the cell surface between TGFβ ligands and various TGFβRII isoforms as suggested by del Re et al.[32] The precise mechanism whereby TGFβ ligand binding may induce receptor activation is conflicting. Some models propose that TGFβ ligands bind TGFβRII dimers that recruit TGFβRI dimers to form a heterotetrameric signaling complex.[35] Other models, which propose the existence of inactive preformed complexes of TGFβRI and TGFβRII dimers,[36,37] are supported by potential cooperative TGFβ2 ligand binding to a TGFβRI-TGFβRIIb complex in which the receptors make physical contact.[32] Krishnaveni et al[38] suggest that TGFβRIIb favors heterodimerization with TGFβRII because this interaction is more robust. Overall, these data suggest a complex TGFβ signaling process further depending on the stoichiometric interactions between TGFβ ligands and various receptor isoforms. Further investigation in vivo of these interactions will add to our understanding of aortic aneurysm pathogenesis.

Aberrant TGFβ signaling that results from type I and II receptor mutations has been implicated in the pathogenesis of cardiovascular disorders involving TAAs. We showed that TGFβ2 signaling is decreased in cells expressing TGFβRIIb, and mutations in this receptor result in increased TGFβ2 signaling. Identification of *TGFβRIIb* activating mutations in 2 TAA patients supports the hypothesis that an increase in TGFβ signaling contributes to aortic pathogenesis. Furthermore, this evidence highlights the scientific and clinical import of expanding diagnostic strategies to include the alternative segment of *TGFβRIIb* in genetic screening of individuals with TAA. Taken together, these findings suggest that TGFβRIIb expression is likely an important regulatory mechanism of TGFβ2 signaling in the aorta, and there may be differential contributions of TGFβ1 and TGFβ2 signaling to aneurysm pathogenesis.

Acknowledgments

We are grateful to the family members, cardiologists, and cardiothoracic surgeons at Weill Cornell Medical Center for their participation in this study. We thank Dr Harry Ostrer for contributing the Ashkenazi Jewish control samples.

Sources of Funding

This work was supported by grants from National Institutes of Health (R01 HL61785 [Drs Basson and Hatcher]), the Snart Cardiovascular Fund (Dr Hatcher), and Raymond and Beverly Sackler (Dr Hatcher).

Disclosures

None.

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CLINICAL PERSPECTIVE

Thoracic aortic aneurysm (TAA) is a common progressive disorder involving gradual dilation of the ascending and/or descending thoracic aorta that is clinically unsuspected until potentially lethal aortic dissection or rupture. TAA is often part of a complex connective tissue syndrome such as Marfan, Loeys-Dietz, and Ehlers-Danlos syndromes, but nonsyndromic TAAs also occur as genetically triggered inherited disorders. Genetically triggered TAAs account for ≈20% of TAAs. Prior analyses of familial TAA in research cohorts have identified disease-causing mutations in genes encoding myosin heavy chain 11 (*MYH11*), α-smooth muscle actin (*ACTA2*), and transforming growth β receptors I and II (*TGFβRI* and *TGFβRII*). Mutational analyses of these 4 genes were now performed in a cohort of patients routinely presenting to cardiovascular clinics and suspected to have genetically mediated nonsyndromic aortic disease. Nine percent of patients had a mutation in one of the 4 genes analyzed. Three percent of patients had mutations in *ACTA2*, 3% in *MYH11,* and 3% in *TGFβRII*. *TGFβRII* mutations included ones identified in an alternatively spliced *TGFβRII* exon, exon 1a, that encodes the TGFβRIIb isoform. These *TGFβRII* exon 1a mutations occurred in 2% of these TAA patients and suggest that altered TGFβ2 signaling contributes to aneurysm pathogenesis. This study's findings support the potential value to cardiovascular practitioners of genetic testing with a multigene aortic disease gene panel in the diagnostic workup of TAA patients.