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Sequencing of TGF-β pathway genes in familial cases of intracranial aneurysm

Teresa Santiago-Sim, Ph.D., **Sumy Mathew-Joseph, Ph.D.**, **Hariyadarshi Pannu, Ph.D.**, **Dianna M. Milewicz, M.D., Ph.D.**, **Christine E. Seidman, M.D.**, **J.G. Seidman, Ph.D.**, and **Dong H. Kim, M.D.**

From the Department of Genetics, Harvard Medical School, Boston, MA 02115, U.S.A. (T.S.S., S.M.J., J.G.S., C.E.S.); Department of Internal Medicine and Institute of Molecular Medicine, The University of Texas Health Science Center at Houston, Houston, TX 77030, U.S.A. (H.P., D.M.M.); Department of Pathology, Brigham and Women's Hospital, Boston, MA 02115, U.S.A. (C.E.S.); and Department of Neurosurgery, The University of Texas Medical School at Houston, Houston, TX 77030, U.S.A (D.H.K.).

Abstract

Background and Purpose—Familial aggregation of intracranial aneurysms (IA) strongly suggests a genetic contribution to pathogenesis. However, genetic risk factors have yet to be defined. For families affected by aortic aneurysms, specific gene variants have been identified, many affecting the receptors to transforming growth factor-beta (TGF-β). In recent work, we found that aortic and intracranial aneurysms may share a common genetic basis in some families. We hypothesized, therefore, that mutations in TGF-β receptors might also play a role in IA pathogenesis.

Methods—To identify genetic variants in TGF-β and its receptors, *TGFB1*, *TGFBR1*, *TGFBR2*, *ACVR1*, *TGFBR3* and *ENG* were directly sequenced in 44 unrelated patients with familial IA. Novel variants were confirmed by restriction digestion analyses, and allele frequencies were analyzed in cases versus individuals without known intracranial disease. Similarly, allele frequencies of a subset of known SNPs in each gene were also analyzed for association with IA.

Results—No mutations were found in *TGFB1*, *TGFBR1*, *TGFBR2* or *ACVR1*. Novel variants identified in *ENG* (p.A60E) and *TGFBR3* (p.W112R) were not detected in at least 892 reference chromosomes. *ENG* p.A60E showed significant association with familial IA in case-control studies (P = 0.0080). No association with IA could be found for any of the known polymorphisms tested.

Conclusions—Mutations in TGF-β receptor genes are not a major cause of IA. However, we identified rare variants in *ENG* and *TGFBR3* that may be important for IA pathogenesis in a subset of families.

Keywords

aneurysm; endoglin; betaglycan; *TGFBR1*; *TGFBR2*

Correspondence to Dong H. Kim, M.D.: The University of Texas Medical School at Houston, 6431 Fannin Street, MSB7.146, Houston, TX 77030, U.S.A. Tel: +1 7135006170; Fax: +1 7135000601; E-mail: E-mail: Dong.H.Kim@uth.tmc.edu.

DISCLOSURES

None

Introduction

Familial aggregation of saccular intracranial aneurysms (IA) indicates a genetic role in the pathogenesis of disease [1]. Up to 20% of IA patients have a positive family history [2–5] and the risk of IA rupture is seven-fold higher among first-degree relatives compared to second-degree relatives [6]. Affected pairs of monozygotic twins have been identified [7].

Although more than 500 families affected by IA have been described to date, no single gene variant has been conclusively shown to cause IA formation or rupture, neither in a single family nor a subpopulation of patients. The clinical features of IA have made genetic analyses difficult. Many affected relatives die due to the catastrophic nature of rupture of an IA, therefore making the collection of DNA samples difficult. Aneurysms typically arise late in life, even in the sixth and seventh decades, making the characterization of unaffected relatives uncertain. Most importantly, there is significant genetic heterogeneity associated with this condition, in which different genes or genetic mechanisms may demonstrate variable expression in terms of age of onset or disease penetrance. Several patterns of inheritance have been observed in families with IA and a single Mendelian model of inheritance has not been established [8].

Although many loci have been linked to familial IA risk, disease-causing genetic variants have not been identified in these intervals [1]. Similarly, association studies involving candidate genes, such as elastin, collagen, and matrix metalloproteinases, have not shown definitive results [1].

In some families, the formation of IA may share a genetic predisposition with aortic aneurysms (AA). Kim et al. have reported that in a group of 274 IA patients, 10% had a family history of AA[9]. Furthermore, pedigree analyses suggested that an autosomal dominant inheritance with decreased penetrance and variable expression was likely in some families. Conversely, several families with AA have been described in which some family members were diagnosed with IA[10, 11]. Sharing common risk factors such as hypertension and smoking, IA and AA display similar pathologies including degeneration of the extracellular matrix, destruction of elastic lamina and loss of media. Both show increased activity of matrix metalloproteinases[12, 13], apoptosis[14, 15] and inflammatory cell infiltration[16, 17].

Based on these data, we hypothesized that formation of IA and AA might share common genetic mechanisms. Gene mutations leading to thoracic aortic aneurysms and dissections (TAAD) have been characterized. TAAD can be inherited in isolation (familial TAAD) or in association with genetic syndromes such as Marfan syndrome (MFS) and Loeys-Dietz syndrome (LDS). Heterozygous mutations in TGF-β receptor genes, *TGFBR1* and *TGFBR2*, have been reported in familial TAAD, MFS and LDS[18–21]. The gene that is responsible for most cases of MFS, the *FBN1* gene encoding fibrillin-1, is required for effective TGF-β activation. These suggest that dysregulation of TGF-β signaling might be involved in TAAD pathogenesis. Accordingly, the expression of genes normally stimulated by TGF-β, such as collagen and connective tissue growth factor, was upregulated in tissue of LDS patients. Furthermore, aneurysms that develop in an accepted mouse model of MFS (*fbn1*$^{C139G/+}$) are associated with increased TGF-β signaling and can be prevented by TGF-β antagonists[22].

TGF-β is a polypeptide that plays diverse roles in cell proliferation and differentiation, apoptosis, and extracellular matrix formation[23, 24]. TGF-β transduces its signals via types I and II receptors, encoded by *TGFBR1* and *TGFBR2*. The ligand-bound type-II receptor phosphorylates the glycine/serine-rich domain of the type-I receptor, which activates signal transduction.

To test our hypothesis that dysregulation of TGF-β signaling may be common to both TAAD and IA, we sequenced the coding region of *TGFBR1* and *TGFBR2* in 44 unrelated IA patients.

We also sequenced genes encoding the TGF-β1 ligand, another TGF-β receptor (*ACVR1*), and co-receptors endoglin (*ENG*) and betaglycan (*TGFBR3*). We report the absence of mutations in *TGFBR1* and *TGFBR2* and the identification of novel variants in *ENG* and *TGFBR3*.

Materials and Methods

Study Population

Between July 2000 and December 2002, 378 patients with saccular IA treated by the senior author (DHK) were eligible for enrollment. Excluded were 56 patients with dissecting or fusiform aneurysms, or patients with associated abnormalities such as an arteriovenous malformation. Enrollment was completed in 274 patients (85%). This cohort does not represent a population-based study and referral bias is a possibility because the patient population was referred to a single surgeon.

Among the study participants were 73.5% female and 26.5% male. The ethnic background was Caucasian in 61.5%, African-American in 18.9%, and Hispanic in 16.4%. The average age of the patients was 53.7 years. There was no difference in the age of presentation between men and women (52.1 and 54.3 years, respectively) or between ethnic groups.

When a patient had a first-degree relative with either an IA or AA, that family was identified as having a possible familial aneurysm (defined as two or more affected first degree relatives). In such families, all relatives were also approached to participate in the study.

If family members reported an aneurysm or a history suggestive for aneurysm such as “stroke” or “sudden death,” the diagnosis was confirmed with medical records, death certificates, or autopsy reports before a positive finding was noted. When the aneurysm history could not be confirmed, the family member was not noted as having an aneurysm. Patients with infundibular enlargements were not classified as having IAs.

Of the 274 patients enrolled, 79 patients (28.8%) had a family history of aneurysms (familial cases) and the remaining 195 (71.2%) did not have any family history of aneurysms (sporadic cases). Of the familial cases, 50 patients (18.2%) had a family history of IA only, while 29 (10.6%) had a family history of both IA and AA. Of the 50 patients with family history of IA only, 6 had polycystic kidney disease (ADPKD), known to increase the risk of IA development.

Families with history of both AA and IA, and families affected by ADPKD (where the gene defects are known), were excluded from our studies. Hence, our study population consisted of 44 probands from families with history of IA only (70.5% Caucasian, 18.2% African-American, 6.8% Hispanic 4.5% and Asian; ages 28–92 years, average of 55 years; 75% female, 25% male). Ninety-two randomly-selected sporadic patients were also analyzed (100% Caucasian; age 24–84 years, average of 54 years; 67% female, 33% male).

For the evaluation of allele frequencies of novel variants in the general population, we used a group of 492 unrelated individuals (we termed “General Population I”) without known intracranial disease (42.7% Caucasian, 21.7% African-American, 3.9% Hispanic, 1.6% Asian, and 30.1% unknown). Of the 492 individuals, 192 specified their age (16–95, average of 50 years) and 396 specified their gender (52.5% female, 47.5% male). No diagnostic tests were performed on these individuals to exclude the presence of IA.

This study was reviewed and approved by the Institutional Review Boards at the University of Texas Health Science Center at Houston and the Brigham and Women’s Hospital in Boston. All information gathered was coded and confidentiality maintained.

DNA Sequencing

Genomic DNA was isolated from peripheral blood or buccal cells using DNA purification kits (PureGene and Oragene). DNA amplifications were done using intron-based, exon-specific primers (Table 1) using the following conditions: 95°C for 10 min; 30 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 1 min, and a final 72°C for 10 min. For most reactions, the forward M13 universal primer tag was appended to the 5' end of the forward primer, and the reverse M13 primer tag to the 5' end of the reverse primer, allowing all forward and reverse sequencing reactions to be performed using the forward and reverse M13 primers, respectively. Sequencing reactions were performed using Big Dye chemistry under the following conditions: 96°C, 10 sec; 50°C, 5 sec; 60°C, 4 min. The products were purified using CleanSEQ magnetic beads (Agencourt) and analyzed on the ABI3100 Genetic Analyzer (Applied Biosystems). Mutation analyses were done by visual inspection of aligned sequences in comparison with published genomic DNA sequences using Sequence Manager 6.1 (DNA Star) and Mutation Surveyor. The NCBI SNP database (dbSNP; http://www.ncbi.nlm.nih.gov/SNP/) was used as reference for identifying known polymorphisms.

Restriction Digestion Analysis

For probands containing novel variants, amplified exons were digested with restriction enzymes. DNA samples from General Population I were used as negative control. PCR reactions were performed using r*Tth* polymerase system (GeneAmpXL Kit, Applied Biosystems). The following conditions were used to amplify the *TGFBR3* exon 4: 96°C, 2 min; 35 cycles of 95°C, 20 sec; 60°C, 30 sec; 72°C, 30 sec; and a final 72°C, 5 min. The same conditions were used for the other exons, except that annealing temperatures were at 55°C for *TGFBR3* exon 16; and 55°C and 65°C for *ENG* exons 2 and 14, respectively. PCR products were digested with *Hph* I (*TGFBR3* exon 4), *Bsr* DI (*TGFBR3* exon 16), *Mwo* I (*ENG* exon 2) or *Stu* I (*ENG* exon 14) (New England Biolabs). Digestion products were visualized in agarose gels. To confirm *ENG* p.R205W and p.R232W which did not alter a restriction site, independent re-sequencing reactions were performed.

Testing association of Novel Variants with IA

To test the association of novel variants with IA, allelic frequencies were analyzed in cases (familial or sporadic groups) versus individuals from General Population I. Variants were genotyped by sequencing, restriction digestion or by using a MassARRAY genotyping system (Sequenom). Variants detected by restriction digestion were verified by sequencing. P-values were calculated with Fisher's exact test with Bonferroni correction.

Testing association of previously identified SNPs with IA

To further test the association of TGF-β pathway genes with IA, allelic frequencies of selected known SNPs in *TGFB1*, *TGFBR1*, *TGFBR2*, *ACVR1*, *TGFBR3* or *ENG* were compared between cases and reference populations. To avoid population stratification issues, only Caucasian subjects were included in the analyses. We compared a case group of 31 familial probands from the original 44 (77% female and 23% male; ages 28–92 years, average 58 years) against two reference groups, one with 150 Caucasian individuals from General Population I (54% female and 46% male; unknown ages) and the other with 60 unrelated Caucasian HapMap subjects from the International HapMap Project (http://www.hapmap.org/) (50% female and 50% male; unknown ages). These HapMap samples were derived from U.S. residents with northern and western European ancestry collected by the Centre d'Etude du Polymorphisme Humain (CEPH). Genotyping and association testing were done as described above.

Alignment of protein sequences

Information on endoglin and betaglycan protein sequences in humans and in mouse, rat, dog, cow, chicken, zebrafish, pig and orangutan was obtained from the NCBI (http://www.ncbi.nlm.nih.gov) and ENSEMBL (http://www.ensembl.org/index.html) genome browsers. Protein sequences were aligned using the MegAlign program.

Results

TGFB1, *TGFBR1*, *TGFBR2*, *ACVR1*, *TGFBR3* and *ENG* were directly sequenced in 44 familial IA patients using intron-based, exon-specific primers. A total of 85 variants, including single nucleotide substitutions, deletions and insertions were identified, mostly located in closely-flanking intronic regions (Appendix Table).

Seven novel coding region variants were identified, two of which were silent amino acid substitutions in *TGFB1* and *ACVR1*. The remaining 5 were non-synonymous substitutions in *ENG* and *TGFBR3* (Table 2). No novel variant was detected in *TGFBR1* or *TGFBR2*. Furthermore, none of the *TGFBR1* or *TGFBR2* mutations previously reported MFS, LDS or TAAD was detected in these patients.

Novel non-synonymous variants were genotyped in a group of individuals without known intracranial disease. *ENG* p.L587F and *TGFBR3* p.I790F were found in 1/892 and 3/872 chromosomes, respectively, whereas *ENG* p.A60E, *ENG* p.R205W and *TGFBR3* p.W112R were not detected in these individuals (Table 2). These variants were also genotyped in 92 sporadic IA patients, where *TGFBR3* p.I790F was detected in 1/184 chromosomes. Allele frequency data suggest that p.A60E is significantly associated with IA in familial cases but not with IA in sporadic cases.

Sequence analyses of *ENG* and *TGFBR3* in sporadic cases revealed another novel variant in *ENG*, p.R232W, which was detected in one case (0.55% allele frequency). However, it was also detected in 3 of 480 control chromosomes (0.63%).

Families with p.A60E, p.R205W or p.W112R were genotyped and clinically screened for IA. In pedigree MG02707, p.R205W was absent in the proband's affected son, and is, therefore, not disease-causing (data not shown). Unfortunately, we are unable to show whether p.A60E or p.W112R co-segregates with the IA phenotype due to the limited number of affected individuals and family members who are willing to participate (Figure 1A–C).

Sequence analyses of human *ENG*/endoglin and *TGFBR3*/betaglycan proteins showed that alanine 60 in endoglin is evolutionarily conserved and is located in the N-terminal region of the protein's extracellular domain (Figure 2A). In p.A60E, a neutral alanine is replaced by a larger, highly-charged glutamate residue. Similarly, tryptophan 112 in betaglycan is highly conserved and is located in this protein's extracellular domain, particularly in a characterized TGF-β binding site (Figure 2B). In p.W112R, a hydrophobic tryptophan is replaced by a hydrophilic arginine residue. To assess the effect of *ENG* p.A60E and *TGFBR3* p.W112R, we applied the web-based tool Polyphen (http://genetics.bwh.harvard.edu/pph/), which was developed to predict whether a missense variant is likely to affect protein structure and function. Based on sequence annotation and alignment, p.A60E was predicted as "possibly damaging" (PSIC score difference: 1.623) and p.W112R as "probably damaging" (PSIC score difference: 3.543).

Allele frequencies of a subset of previously identified SNPs in *TGFB1*, *TGFBR1*, *TGFBR2*, *ACVR1*, *TGFBR3* and *ENG* were analyzed in cases versus individuals without known intracranial disease (Table 3). P-values less than 0.05 were obtained for rs1155705

(*TGFBR2*) and rs1146031 (*ACVR1*). However, after applying Bonferroni correction, P-values were not statistical significant.

Discussion

Mutations in TGF-β receptors, *TGFBR1* and *TGFBR2*, are associated with thoracic aortic aneurysms and dissections (TAADs). Previously, we reported that IA and AA segregate in a subset of families and are likely inherited in an autosomal dominant fashion. In this study, we tested the hypothesis that IA and AA share a common genetic basis in some families. Specifically, we hypothesized that mutations affecting TGF-β signaling might also play a role in IA pathogenesis. We sequenced 44 familial IA patients to identify variants in the *TGFB1* ligand, signaling receptors and co-receptors. No novel missense variants were found in *TGFBR1* and *TGFBR2*. Furthermore, none of the previously identified mutations associated with TAAD were detected, suggesting that mutations in *TGFBR1* and *TGFBR2* are not a major cause of IA development. However, novel variants were identified in *ENG*/endoglin and *TGFBR3*/betaglycan in a small subset of patients.

Endoglin and betaglycan are transmembrane proteins that modulate TGF-β-mediated cellular responses. They have large extracellular domains and serine/threonine-rich cytoplasmic regions. The importance of endoglin in vessel wall structural development has been demonstrated by defective vasculature and lethality in an endoglin knockout mouse model. In humans, endoglin mutations are associated with hemorrhagic telangiectasia 1 (HHT1), an autosomal dominant disorder characterized by altered vascular development resulting to capillary telangiectasias and arteriovenous malformations of the skin, lung, liver, gastrointestinal tract and brain. With the recognized role of endoglin in TGF-β signaling, it is thought that abnormal vessel development in HHT1 is due to abnormal TGF signaling during vascular development[25]. At present, there are more than one hundred endoglin mutations identified in HHT1 patients, all involving the extracellular domain, including deletions (~33%), missense (20%), insertions (~16%), nonsense (~15%), splice site mutations (~13%), and indels (~3%)[26]. In most cases, these mutations lead to unstable transcripts and reduced levels of functional mutant proteins, suggesting that haploinsufficiency is the major underlying mechanism for HHT1[26]. In some HHT cases, additional vascular complications may arise, such as aneurysms in various organs, including the brain[27]. In our study, we identified a novel *ENG* variant (p.A60E) that has not been described in HHT.

Betaglycan has been implicated in various developmental processes. Knockout mouse models demonstrate a defect in coronary vasculogenesis [28] and in heart and liver development [29]. In humans, betaglycan has been implicated in tumor suppression and ovarian function regulation[30, 31]. The novel betaglycan variant we noted, p.W112R, has not been described previously. Both betaglycan p.W112R and endoglin p.A60E affect highly conserved residues and were absent in hundreds of reference samples tested, suggesting their importance in protein function. However, studies involving additional affected families and functional analyses are needed to elucidate their role in specific role IA pathogenesis.

The role of endoglin in IA susceptibility has been tested by other investigators and conflicting results have been reported. A 6-base insertion polymorphism in endoglin showed association with IA in a Japanese population [32], but this result could not be replicated in a Caucasian, Korean and another Japanese population [33–35]. SNP rs1800956 showed association with IA in a Korean population[35], but not in a Japanese population[34]. In our study, both polymorphisms failed to associate with IA. These results suggest that endoglin, like *TGFBR1* and *TGFBR2*, is not a major IA susceptibility gene. However, it is possible that elucidation of the effects of variants can shed light on the pathogenetic mechanisms underlying IA formation.

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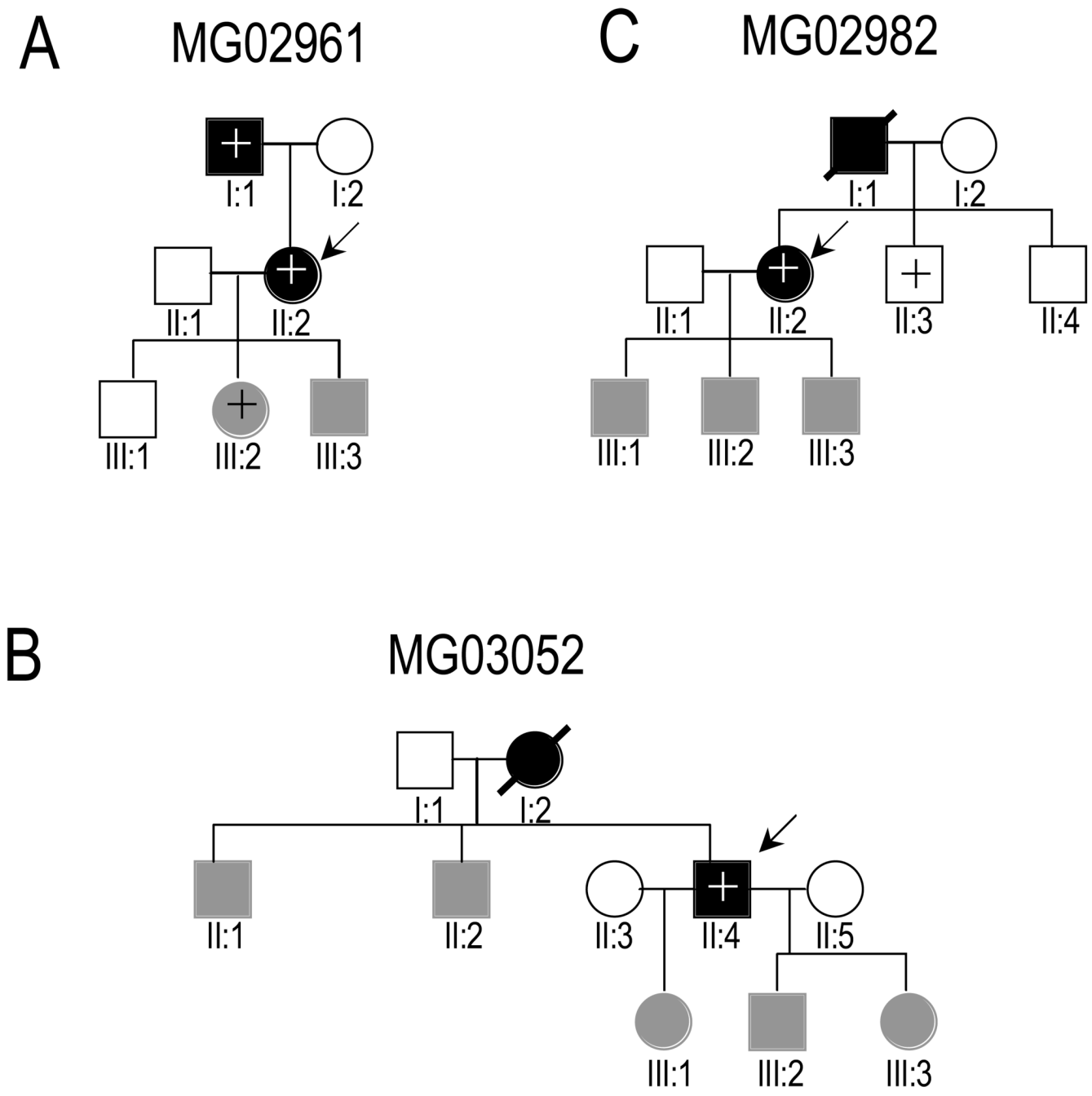


Figure 1.
Pedigrees of families with *ENG* p.A60E (A and B) or *TGFBR3* p.W112R (C). Arrows indicate probands. IA-affected, unaffected and clinically unscreened individuals are indicated by blackened, open and grayed symbols, respectively. Presence or absence of the variant, determined by sequencing or restriction digestion analysis, is indicated by + or −, respectively.

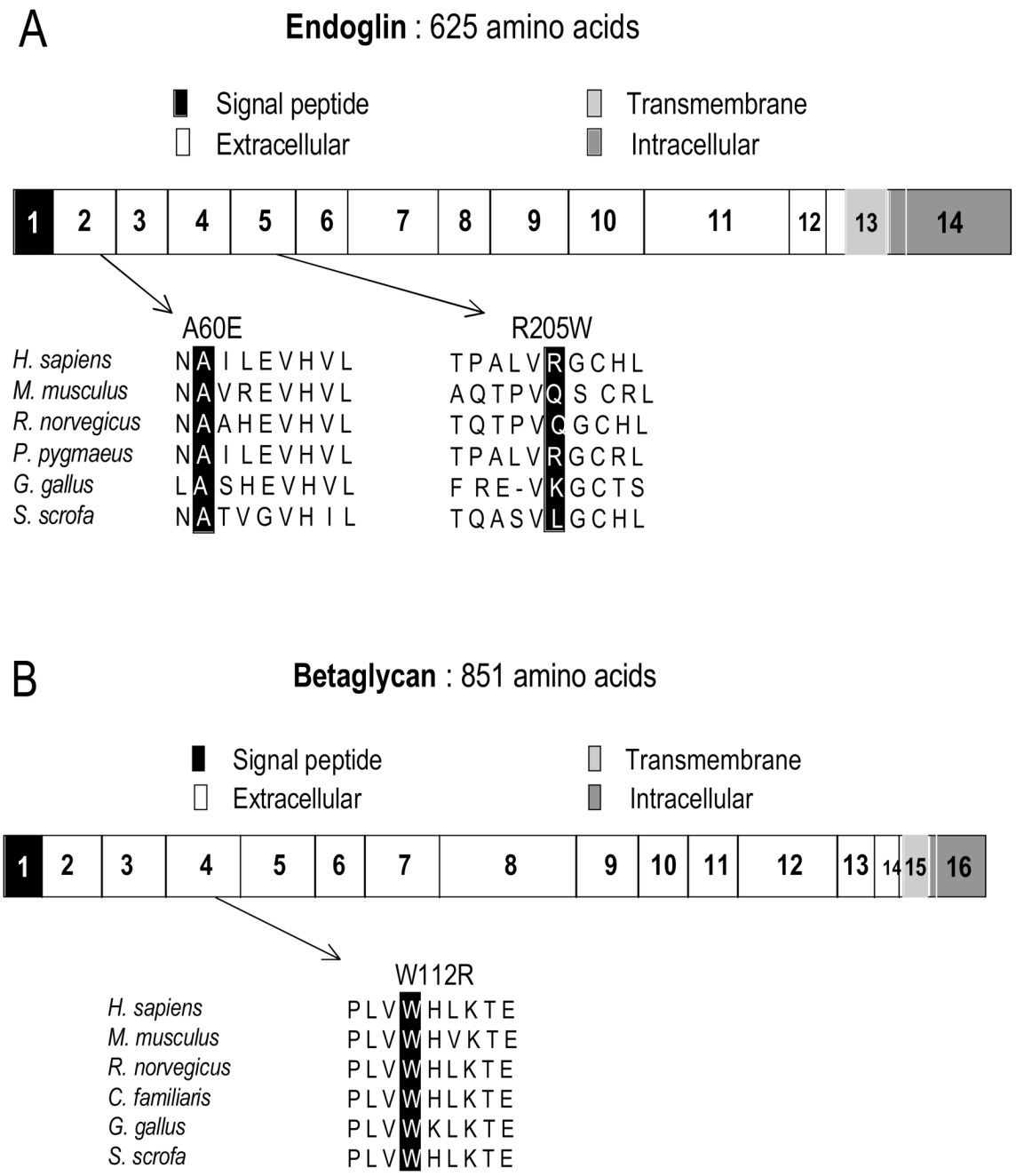


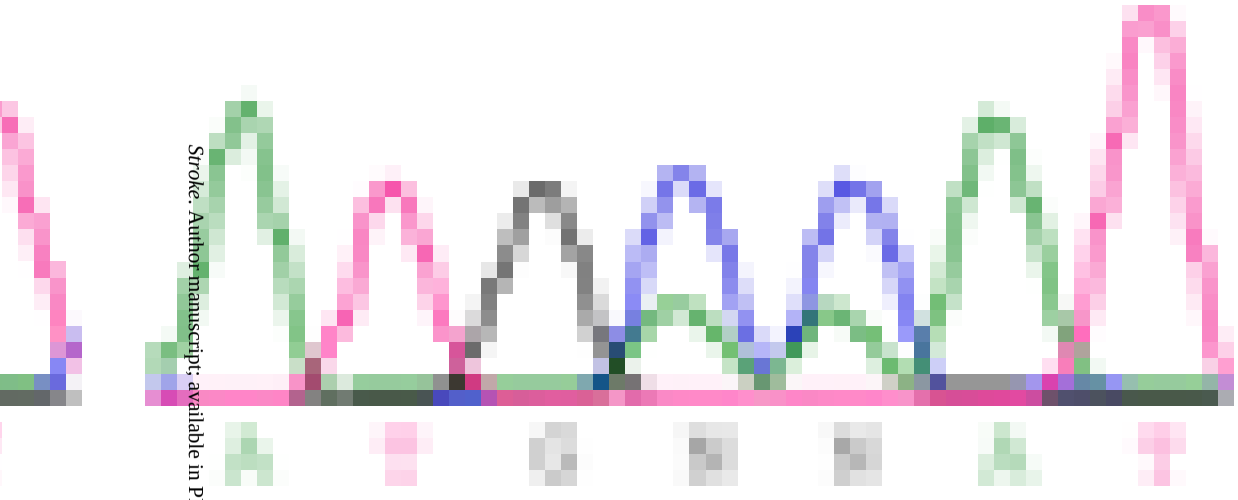
Figure 2.

Schematic diagram of human endoglin (A) and betaglycan (B) genes. Variants are shown in relation to exons (numbered) and domain organization. Accession numbers, Endoglin: *H.sapiens* NP_000109; *M.musculus*, NP_031958; *R.norvegicus*, NP001010968; *P.pygmaeus*, CAH92389; *G.gallus*, AAT84715; *S.scrofa*, NP_999196. Betaglycan: *H.sapiens*, NP_003234; *M.musculus*, AAC28564; *R.norvegicus*, NP_058952; *C.familiaris*, XP_547284; *G.gallus*, NP_989670; *S.scrofa*, NP_999437.

Table 1
Oligonucleotides used in this study

		Forward primers (5' -> 3')	Reverse primers (5' -> 3')
A. Oligonucleotides used for sequencing of coding regions			
TGFB1	Exon 1	GAGGACCTCAGCTTTCCCTC	GCCAGTTTCTTCTGCCAGTC
	Exon 2	TCAGAGACTGACTCCACCCC	TTCAGGGACCATCTAGGTGG
	Exon 3	TTTTTCTCCACCCCTCCTCT	ATCACTCAGGTTTCCATGCC
	Exon 4–5	AGAGAGCTGCAGTGAGAGGG	AGCCCTCCAAGCTAAAGGAG
	Exon 6	AGGGAGACCCAGATGGAGAT	CCCTCTCTAGCTTCCTGCCT
	Exon 7	CGAATTGGAGATGGGAAGAG	ATCAGAGTCCCTGCATCTCA
TGFBR1	Exon 1	CCTCCGAGCAGTTACAAAGG	GCGCCATGTTTGAGAAAGAG
	Exon 2	TGCTACATTTCCTTGGGCTT	AACACATACCCAGGAGGCAG
	Exon 3	CTGACAGAGCTGGTGTGCAT	GGAGCTGACTTATTGATTCGC
	Exon 4	CCCTCTAGCAGGAGTTTCTGG	AGGAATGCTATCAAGAGTCAAGA
	Exon 5	TTGCAGTGTGTGACTCAGGA	GATGCGGTTTTGTCATGTTG
	Exon 6	GTGGGCTGAAATGCTTTGAT	ATTTTCTGGAAGGGCAACCT
	Exon 7	AGATCATGAGGCAGATAGTGTG	GCCTTTGTTTTCTCTGGCAC
	Exon 8	GGAAGTGGCTTGTGGATACAG	AAAGGCCACTGCAAATGTTC
	Exon 9	TCTTTGTCCACCTGCTTTCC	AGTGCACAGAAAGGACCCAC
TGFBR2	Exon 1	GGACTCCTG TGCAGCTTCC	CACAATCCCTGCAGCTACG
	Exon 1a	AACTTTGAAGAAAACATATTGACCA	AAGCAGTGAGGGAGCATGAC
	Exon 2	TGAAATTGCATAACATCTTCAGG	GGAAAGGGAAATGGAACAGG
	Exon 3	CAGATTGCCTTTCTGTCTGGA	CCACAGGAGGAATGTGCTCT
	Exon 4a	GCACTTGCATCCCTGAAATAA	ACCTCAGCAAAGCGACCTT
	Exon 4b	GGAAGATGACCGCTCTGACA	ACTGTGGAGGTGAGCAATCC
	Exon 4c	GGGGAAACAATACTGGCTGA	TTCCCTAGACCAGTG TCCAGA
	Exon 5	AGGGGCCACCATCAGCTA	CCCTGGAATAATGCTCGAAG
	Exon 6	AGCCAGGCATCTCACCAT	CAGGGCCATAGAACACAATG
	Exon 7	GACCCTGCTTGCACTCACTA	TCTGCTTATCCCCACAGCTT
TGFBR3	Exon 1	TTTGAAAATTGCAAGGAGGG	CATGCTAGGAGGCCAAGAAG
	Exon 2	GCAGTGGTTTGATCACCCTT	AAGTTGCCCAATCCAGACAG
	Exon 3	TTCTTTGGCAGGGAGCTAAA	AAGCTCAGGCCACACAGAGT
	Exon 4	GAATCCGCTGCTTAAAAACG	GTGATTCCTTGCCCTAACCA
	Exon 5	GTCAATAGGCGGTCACTGGT	GATGAAGCACACCTGAAGCA
	Exon 6	TGAAGACTTGGAAGAGGGGA	GGGTTCAGAGAGGTTAGGGG
	Exon 7	TCATCGTTCTTAGCCCAAGG	CTGGAAAAGCTTCATTTGGG
	Exon 8	ACACCTGCCCATTCTATGCT	GTAGGCCCATCCAACTGAGA
	Exon 9	GAATTTCCAAGGCCAACAGA	TTGACAGTGCAGCCTTTGTC
	Exon 10–11	GCAGAACCAAACACACATGG	GGTCTGTGAGGTAGGACAGGA
	Exon 12	AAAGCAGCGTGTCATCTGAA	GGGCAGTTCCAAAACAAAAA
	Exon 13	AGGTAGAGCTGGTGAAGGCA	GAATGCAAGGGAGAGTGACC
	Exon 14	AGTTTAGGTGTTTGCCTTCA	CTACCTTCCCATTCAAGCCA
	Exon 15	GAATCTTCATTGCATTCTCCG	TTCACCAACATCACAATCGC
	Exon 16	AGTTAAAATGGCAAATGCGG	TCATGTTTATACTAGCCCTGGG

		Forward primers (5' -> 3')	Reverse primers (5' -> 3')
ENG	Exon 1	TCCCTGTGTCCACTTCTCCT	CCGAGGCTTTCTTTCAACAC
	Exon 2	TAAGGTGGCTGTGATGATGC	TCAGCTCTTCCCACCTGAGT
	Exon 3	TGGAAGCATCCAAATCATCA	CATCAACCTGACTCCCACCT
	Exon 4	GGCTGATCTGACTGCTAGGG	GATATTTGGTGGAGGAGCCA
	Exon 5	CCACCACTATCTTTGGCTGT	GGCTTTATAAGGGACCGGAG
	Exon 6	CTCCGGTCCCTTATAAAGCC	CCTTGCCCAAGCTCACA
	Exon 7	AACCCAAACTCCCAACCTCT	ATCTTGGCTCACTGCAACCT
	Exon 8	GGGCACACAGTGATCACACA	CCACATCTTACTGTGCCACG
	Exon 9–10	CTGGGTTGTGGTCAGTCCTT	CATTCCAGACACACATGGCT
	Exon 11	GAGTCAGGCAACTCCACAGG	AAGAGTTCCCACCCCTGAGT
	Exon 12	TGCTCAGGGACACTGACAAG	AGGCCACATGCCTGATTAAG
	Exon 13	AGTGTTCACAAGGGTGAGGG	CTAGGCTGCTATGGCTCTGG
	Exon 14–15	TTCAGAGAAGTCGAGGGTCC	TGAGTTCACACCAGTGCTCC
ACVR1	Exon 1	TGAATGGCAGTTTGAAGGTG	CTCCCAAAGTGCTGGGATTA
	Exon 2	TCATGGTTGATGGTGATGCT	CCAGGGTGACCTTCCTTGTA
	Exon 3	TGTGTGGTCAGGATCAGGAG	GGGAAGACTACACAGGTGCC
	Exon 4	GGTTGCAGTGAACCCAGATT	TCTCTCATCATCCCAAAGGG
	Exon 5	GGGCAGCTTCCACCTTATTT	CAAAACGGAGAGAGCAAAGG
	Exon 6	CCTCTTAGGGCAATTGGTCA	AACATGTTGTGGGGGAGAGA
	Exon 7	AGTGACCCTGGATCCACAAG	AATGGCTGGTCTCTTCCAGA
	Exon 8	ATTGCCTTTTTCTCCCACCT	AGATCCACGGGACAGATCAC
	Exon 9	CCAATCTGGCCTATGTCGTT	AGCGAGGTTAGGGTGGTTTT
B. Oligonucleotides used for restriction digestion analysis			
TGFBR3	Exon 3	TCTGTTGATAGTGAGTTGCAAAAA	ACACCCGGTAGCCAGTTACA
	Exon 14	AGTTTAGGTGTTTGCCTTCA	CTACCTTCCCATTCAAGCCA
	Exon 15	TCTTCATTGCATTCTCCGATT	TTTTGTGAAACCCAATTTATACCA
ENG	Exon 1	GCGTCCCTGTGTCCACTT	CCGAGGCTTTCTTTCAACAC
	Exon 2	AATCCATGGAACGAATATAATGA	AGACCCTGCCCCTAGAAATG
	Exon 14	AGGCCTTGGCTGTGATGAG	GCTGCTCAGTCTCTCCTGCT

DNA Sequence Trace	Variant	Minor Allele Frequency			Fisher's Exact P-value[†]	
		Fam IA (n_m/n_{chr})*	Spor IA (n_m/n_{chr})*	GPI (n_m/n_{chr})*	Fam IA vs GPI*	Spor IA vs GPI*
		0.023 (2/88)	0.000 (0/182)	0.000 (0/892)	0.0080	
		0.011 (1/88)	0.000 (0/184)	0.000 (0/482)	0.1544	

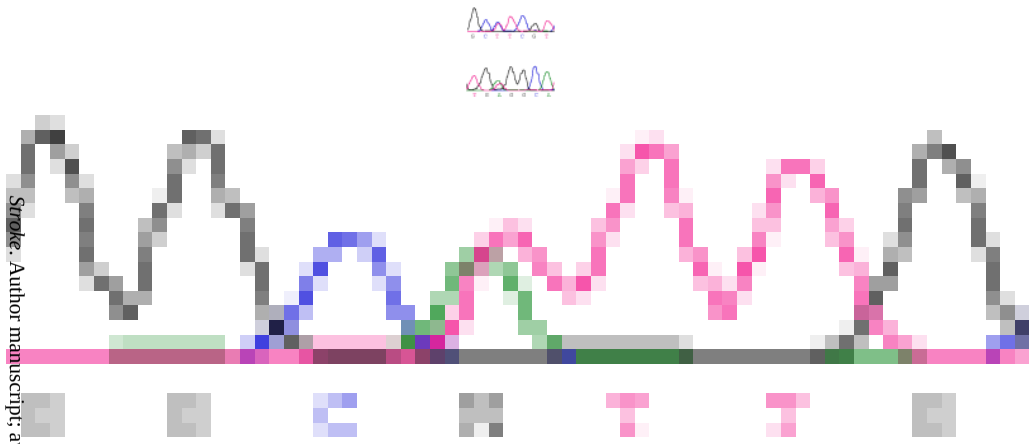
DNA Sequence Trace	Variant	Minor Allele Frequency			Fisher's Exact P-value[†]	
		Fam IA (n_m/n_{chr})*	Spor IA (n_m/n_{chr})*	GPI (n_m/n_{chr})*	Fam IA vs GPI*	Spor IA vs GPI*
		0.011 (1/88)	0.000 (0/182)	0.001 (1/892)	0.1716	
		0.011 (1/88)	0.000 (0/184)	0.000 (0/900)	0.0891	
		0.011 (1/88)	0.005 (1/184)	0.003 (3/872)	0.3197	0.1423

Table 3

Allele Frequencies of Known SNPs in IA patients versus the General Population

Gene	SNP ID	Location	Variation M/m*	Minor Allele Frequency		Fisher's Exact P-value
				Familial IA (n_m/n_{chr})†	Reference Population (n_m/n_{chr})†	
A. Using General Population I as reference population‡						
TGFBR1	rs7861780	EX 6	A/C	0.015 (1/66)	0.000 (0/296)	0.1823
TGFBR2	rs11466512	INT 4	T/A	0.288 (19/66)	0.279 (83/298)	0.8804
	rs2228048	EX 5	C/T	0.045 (3/66)	0.017 (5/298)	0.1614
TGFBR3	rs1805109	5'UTR	G/A	0.106 (7/66)	0.065 (19/294)	0.2891
	rs17881268	INT 2	C/T	0.000 (0/66)	0.034 (10/298)	0.2190
	rs1805113	EX 13	T/C	0.439 (29/66)	0.393 (117/298)	0.4908
	rs284878	EX 14	C/T	0.045 (3/66)	0.047 (14/300)	1.0000
	rs17882828	EX 15	G/C	0.030 (2/66)	0.003 (1/298)	0.0859
ENG	rs7847860	INT 2	G/T	0.076 (5/66)	0.064 (19/298)	0.7834
	Ref 32	INT 7	-/GGGGGA	0.242 (16/66)	0.168 (49/292)	0.1602
	rs3739817	EX 8	C/T	0.091 (6/66)	0.070 (21/298)	0.6031
	rs1800956	EX 8	G/C	0.000 (0/66)	0.003 (1/298)	1.0000
ACVR1	rs2227861	EX 5	T/C	0.212 (14/66)	0.253 (75/296)	0.5307
B. Using CEPH-derived HapMap Samples as reference population§						
TGFB1	rs8179181	INT 5	C/T	0.242 (15/62)	0.339 (40/118)	0.2333
TGFBR1	rs334354	INT 7	G/A	0.258 (16/62)	0.217 (26/120)	0.5793
	rs868	3'UTR	A/G	0.258 (16/62)	0.217 (26/120)	0.5793
TGFBR2	rs1155705	INT 3	A/G	0.172 (10/58)	0.317 (38/120)	0.0482
TGFBR3	rs1805109	5'UTR	G/A	0.097 (6/62)	0.085 (10/118)	0.7880
	rs1805110	EX 2	C/T	0.097 (6/62)	0.092 (11/120)	1.0000
	rs2810904	EX 3	G/A	0.274 (17/62)	0.336 (39/116)	0.4983
	rs4658261	INT 10	G/A	0.145 (9/62)	0.178 (21/118)	0.6761
	rs4658260	INT 10	C/T	0.145 (9/62)	0.175 (21/120)	0.6776
	rs1805113	EX 13	T/C	0.435 (27/62)	0.508 (60/118)	0.4328
	rs2296621	INT 14	C/A	0.210 (13/62)	0.250 (30/120)	0.5856

Gene	SNP ID	Location	Variation M/m*	Minor Allele Frequency		Fisher's Exact P-value
				Familial IA (n_m/n_{chr})†	Reference Population (n_m/n_{chr})†	
	rs1805115	3'UTR	G/A	0.177 (11/62)	0.217 (26/120)	0.5669
ENG	rs3739817	EX 8	C/T	0.097 (6/62)	0.075 (9/120)	0.5845
	rs1330684	INT 12	G/A	0.387 (24/62)	0.325 (39/120)	0.4157
	rs10760503	INT 14	T/C	0.435 (27/62)	0.379 (44/116)	0.5216
ACVR1	rs1146031	EX 8	A/G	0.113 (7/62)	0.017 (2/120)	0.0080

*M, major allele; m, minor allele

†n_m, occurrence of minor allele;. n_{chr}, number of chromosomes

‡Significant P-value after Bonferroni correction < 0.003846

§Significant P-value after Bonferroni correction < 0.003125