Association of cystic fibrosis genetic modifiers with congenital bilateral absence of the vas deferens

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Objective: To investigate whether genetic modifiers of cystic fibrosis (CF) lung disease also predispose to congenital bilateral absence of the vas deferens (CBAVD) in association with cystic fibrosis transmembrane conductance regulator (CFTR) mutations. We tested the hypothesis that polymorphisms of transforming growth factor (TGF)-β1 (rs 1982073, rs 1800471) and endothelin receptor type A (EDNRA) (rs 5335, rs 1801708) are associated with the CBAVD phenotype.

Design: Genotyping of subjects with clinical CBAVD.

Setting: Outpatient and hospital-based clinical evaluation.

Patient(s): DNA samples from 80 subjects with CBAVD and 51 healthy male controls from various regions of Europe. This is one of the largest genetic studies of this disease to date.

Intervention(s): None.

Main Outcome Measure(s): Genotype analysis.

Result(s): For single nucleotide polymorphism (SNP) rs 5335, we found increased frequency of the CC genotype among subjects with CBAVD. The difference was significant among Turkish patients versus controls (45.2% vs. 19.4%), and between all cases versus controls (36% vs. 15.7%). No associations between CBAVD penetrance and polymorphisms rs 1982073, rs 1800471, or rs 1801708 were observed.

Conclusion(s): Our findings indicate that endothelin receptor type A polymorphism rs 5335 may be associated with CBAVD penetrance. To our knowledge, this is the first study to investigate genetic modifiers relevant to CBAVD. (Fertil Steril® 2010;94:2122–7. ©2010 by American Society for Reproductive Medicine.)

Key Words: Congenital bilateral absence of the vas deferens, CBAVD, CFTR, cystic fibrosis, CF, modifier gene, TGF-β, EDNRA

Cystic fibrosis (CF) is a common autosomal recessive disorder among whites, and affects 1 in 3,500 live births in the United States (1). The disease is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene (2–4). Cystic fibrosis is characterized by lung, pancreatic, liver, and other exocrine glandular abnormalities. Approximately 98% of men with CF are infertile, and lack the vas deferens bilaterally (5). More than 1,500 disease-causing CFTR mutations have been identified (6). Certain mutations have also been implicated in a variety of CFTR-related pathologic conditions such as disseminated bronchiectasis, allergic bronchopulmonary aspergillosis, diffuse panbronchiolitis, recurrent idiopathic pancreatitis, giant nasal polyposis, and congenital bilateral absence of the vas deferens (CBAVD) (7–9).

Congenital bilateral absence of the vas deferens is associated with normal spermatogenesis and obstructive azoospermia due to lack of the vas (10). A small subset of men without known CFTR defects exhibit CBAVD. However, 80%–97% of subjects with CBAVD posses at least one defective CFTR allele, and 50%–93% of individuals with CBAVD carry two variants, including class IV or V CFTR abnormalities (11–15). Congenital bilateral absence of the vas deferens thus belongs to a group of CF-related disorders—and is considered an isolated, urogenital form of CF. Although a high percentage of patients with CBAVD carry mutations in CFTR, approximately 1 in 29 white men in the United States carries one CFTR variant but never develops CBAVD (16). The finding of a single CFTR mutation is therefore a poor predictor for involution of the vas. Other genetic or environmental factors must modify penetrance of CBAVD, but these are not yet known.

The best characterized CBAVD-specific variant is the polymorphic polythymidine tract (Tn) in CFTR intron 8 (IVS8) for which length is inversely correlated with the degree of exon 9 skipping during messenger RNA (mRNA) splicing. The number of thymidines varies in this tract between 5 and 9, but extremely short alleles

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TABLE 1

Distribution of CFTR mutations among congenital bilateral absence of the vas deferens samples.

Portuguese	CFTR alleles	Spanish	CFTR alleles	Turkish	CFTR alleles
5T	22	F508del	11	5T	20
F508del	14	5T	9	D1152H	14
R334W	5	D443Y[a]	3	D110H	3
R117H	3	G576A[a]	3	F508del	2
S1235R	3	R668C[a]	3	3041-11del7	2
N1303K	2	G542X	2	1767del6	2
P205S	2	R117H	2	2789+5G>A	2
D614G	2	V232D	2	CFTRdele2(ins186)	2
G542X	1	L997F	1	3120+1G>A	1
L206W	1	H609R	1	G1130A	1
V562I	1	N1303H	1	M952I	1
I507del	1	L206W	1	365insT	1
3272-26A>G	1	3272-26A/G	1	E585X	1
2789+5G>A	1	L15P	1	2752-15C>G	1
G576A[a]	1	R347H	1	R334Q	1
R668C[a]	1	2689insG	1	R347H	1
CFTRdele2,3	1	R1070W	1	E831X	1
L1227S	1	I 1027T	1	R1070W	1
E831X	1			3272-26A>G	1
				L997F	1
				I853F	1
				A349V	1
				6T	1

Note: CFTR = cystic fibrosis transmembrane conductance regulator.
[a] in *cis*.

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(with 3 or 2 thymidines) have also been described in CBAVD (17, 18). Lower numbers of thymidine residues in the tract predict an increasing proportion of nonfunctional CFTR (i.e., lacking exon 9) (15). Mak et al. (19) showed that a patient with CBAVD with the common F508del mutation and an IVS8-5T variant produced 32% of the normal levels of CFTR in the lung (exon 9 intact; a level of expression sufficient to maintain a normal pulmonary phenotype), but insufficient full-length CFTR (26% in reproductive tissues) to allow proper structural development of the vas. The amount of functional CFTR (with exon 9) in a CF F508del/IVS8-7T carrier male (38%) in the reproductive tract was suggested to be sufficient for normal function and vas development (19). A "mild" CFTR allele that maintains partial ion channel activity, R117H, is associated with the 5T allele in CF and 7T in CBAVD (20). Rave-Harel et al. (21) examined epithelial tissues from subjects with CBAVD, and showed that levels of normal CFTR transcripts were higher in the nasal epithelium than in epididymal epithelium. Therefore it has been suggested that amounts of CFTR protein required for normal function vary between different tissues (22). In general, the vas deferens has been viewed as a tissue among the most sensitive to altered CFTR activity (22).

Genetic modifiers of the CF pulmonary phenotype represent an area of intensive study. Recently, Drumm et al. (23) showed that the transforming growth factor-β1 (TGF-β1) codon 10 CC genotype (rs 1982073) is associated with severe lung disease among individuals homozygous for CFTR mutations (23). This allele is linked to elevated TGF-β1 gene expression and higher circulating levels of TGF-β1 in human subjects (24–26). A second TGF-β1 single nucleotide polymorphism (SNP) in codon 25 (rs 1800471) may also influence TGF-β1 protein levels (27), and has been implicated as a contributor to CF lung disease progression (27, 28).

In addition to an emerging understanding of TGF-β1 as a modifier of CF severity, Darrah et al. (29) found a strong correlation between lung phenotype in CF and polymorphisms in the endothelin receptor type A (EDNRA) gene. In particular, the genotype AA at position -231 from AUG (rs 1801708) in EDNRA and genotype CC in exon eight (rs 5335) were associated with more severe lung disease in CF women (29). With reference to these results, McKone et al. (30) examined 21 tagSNPs in the endothelin-1, endothelin-3, EDNRA, and EDNRB (endothelin receptor type B) genes. The study confirmed a significant association between an EDNRA haplotype including SNP rs 5335 and CF lung disease, but no association with tagSNPs in other candidate genes (30). Interestingly, both TGF-β and endothelin play a role in extracellular matrix formation (31, 32) wound healing (31, 32), lung diseases such as asthma (33, 34), and lung fibrosis (27, 35–37). In addition, Jain et al. (38) observed a connection between TGF-β and the endothelin–EDNRA system in idiopathic lung fibrosis, and demonstrated that endothelin-1 influences TGF-β1 production through EDNRA.

Based on these considerations, we hypothesized that TGF-β1 and EDNRA polymorphisms might play a role in penetrance of CBAVD. We designed a study to test whether codon 10 or codon 25 TGF-β1 polymorphisms, or either of the two EDNRA gene polymorphisms reported to modify CF lung disease, might also contribute as genetic modifiers of CBAVD.

MATERIALS AND METHODS

Samples

We analyzed genomic DNA samples from 80 individuals with CBAVD and 51 healthy male control subjects. This included 19 patient samples and 20

TABLE 2

EDNRA genotype distributions in patients with congenital bilateral absence of the vas deferens and controls.

EDNRA polymorphisms		Patients		Controls	
Exon 8 (rs 5335)					
Turkish	CC[a]	14	45.2%	6	19.4%
	CG	12	38.7%	20	64.5%
	GG	5	16.1%	5	16.1%
Spanish	CC	4	21.1%	2	10%
	CG	11	57.8%	13	65%
	GG	4	21.1%	5	25%
Total[b]		50		51	
Promoter (rs 1801708)					
Turkish	AA	5	16.1%	8	25.8%
	AG	18	58.1%	10	32.3%
	GG	8	25.8%	13	41.9%
Spanish	AA	0	0	2	10%
	AG	7	36.8%	8	40%
	GG	12	63.2%	10	50%
Total		50		51	

Note: EDNRA = endothelin receptor type A.
[a] $P < .05$ for Turkish population, χ^2 analysis.
[b] $P < .05$ for CC genotype among all subjects shown, χ^2 analysis.

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(non-CBAVD) controls from Spain (Medical and Molecular Genetics Center-IDIBELL), 31 subjects with CBAVD and 31 controls from Turkey (Department of Medical Biology, Hacettepe University), and 30 individuals with CBAVD from Portugal (Department of Genetics, Faculty of Medicine and Laboratory of Cell Biology; Institute Biomedical Sciences Abel Salazar of University of Porto). The study therefore represents one of the largest genetic analyses to date of CBAVD, for which large patient populations are not readily available. Criteria for inclusion as a subject required known CFTR variants. Controls were defined as healthy sperm donors or other unrelated individuals with an intact vas deferens. More than 40 different CFTR polymorphisms of varying "severity" were represented. Because more than 1,500 CF disease-associated mutations have been described previously, it is very likely that other mutations in certain subjects were present but not detected by the genotyping methods described in the present study. The protocol was approved by the Institutional Review Board (IRB) of Human Use at the University of Alabama at Birmingham and by local Portuguese, Spanish, and Turkish ethical committees.

Methods

A 453-bp region of the 5′ end of TGF-β1 gene (GenBank accession number: NT_011109) was amplified using 5′-GAGGACCTCAGCTTTCCCTC-3′ (forward) and 5′-CTCCTTGGCGTAGTAGTCGG-3′ (reverse) primers. This region includes both rs 1982073 and rs 1800471 TGF-β1 SNPs.

TABLE 3

TGF-β1 genotype distribution in patients with congenital bilateral absence of the vas deferens and controls.

TGF-β1 polymorphisms		Patients		Controls	
Codon 10 (rs 1982073)					
Turkish	CC	8	25.8%	13	41.9%
	CT	12	38.7%	8	25.8%
	TT	11	35.5%	10	32.3%
Spanish	CC	4	21.1%	6	30%
	CT	6	31.6%	10	50%
	TT	9	47.4%	4	20%
Total		50		51	
Codon 25 (rs 1800471)					
Turkish	GG	26	83.9%	28	90.3%
	GC	5	16.1%	3	9.7%
	CC	0	0	0	0
Spanish	GG	19	100%	17	85%
	GC	0	0	3	15%
	CC	0	0	0	0%
Total		50		51	

Note: TGF-β1 = transforming growth factor-β1.

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TABLE 4

Allelic frequencies of EDNRA and TGF-β1 SNPs.

	Ethnicity	Patients	Controls
EDNRA exon 8 SNP (rs 5335)			
C allelic frequency			
	Turkish	64.5%	51.6%
	Spanish	50%	42.5%
	Portuguese	33.3%	ND
EDNRA promoter SNP (rs 1801708)			
A allelic frequency			
	Turkish	45.2%	30%
	Spanish	18.4%	41.9%
	Portuguese	26.8%	ND
TGF-β1 codon 10 SNP (rs 1982073)			
T allelic frequency			
	Turkish	54.8%	45.1%
	Spanish	63.2%	45%
	Portuguese[a]	55%	44.4%
TGF-β1 codon 25 SNP (rs 1800471)			
G allelic frequency			
	Turkish	91.9%	95.2%
	Spanish	100%	92.5%
	Portuguese[a]	95%	92.5%

Note: ND = not done; EDNRA = endothelin receptor type A; TGF-β1 = transforming growth factor-β1; SNP = single nucleotide polymorphism.
[a] Control data from Alves H, Histocompatibility Center, University of Porto, Portugal.

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Conditions were as follows: predenaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 45 seconds, and a final extension at 72°C for 5 minutes. A region encompassing 480 bases of the promoter region of EDNRA gene (Ensembl Gene ID: ENSG00000151617), including SNP rs 1801708, was amplified using the primers 5′-GTGGAAGGTCTG-GAGCTTTG-3′ and 5′-TTCCCAGCTCTCGTCTTCTC-3′. Conditions were: 95°C for 5 minutes, followed by 30 cycles of 95°C for 30 seconds, 58°C for 30 seconds and 72°C for 40 seconds. The final extension step was 72°C for 7 minutes. For detection of the exon 8 SNP of the EDNRA gene (rs 5335), we used primers: 5′-CTGCTGCTGTTACCAGTCCA-3′ and 5′-TGACCAGTTCCCATTGAACA-3′ (95°C for 5 minutes, followed by 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 45 seconds, with a final extension step of 72°C for 7 minutes).

Platinum Blue PCR Supermix (Invitrogen, Carlsbad, CA), Apex ™ RED *Taq* DNA Polymerase Mastermix (Genesee Scientific, San Diego, CA), or RedTaq^R DNA polymerase with 10× RedTaq polymerase chain reaction (PCR) reaction buffer (Sigma-Aldrich, Saint Louis, MI) were used for amplification. Because the EDNRA promoter constitutes a GC-rich region, dimethyl sulfoxide (DMSO, 10%) was added to the PCR mixture to increase efficiency of that particular PCR. QIAquick PCR Purification Kit (Qiagen, Valencia, CA) was used before sequence analyses with BigDye Terminator v3.1 Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA). The sequencing products were run by standard protocols on an Applied Biosystems 3730 Genetic Analyzer with POP-7 polymer (Genomics Core Facility of the Howell and Elizabeth Heflin Center for Human Genetics, University of Alabama at Birmingham). Sequence analyses and comparisons were conducted using Chromas Lite software (Chromas Lite Freeware. Tewantin, Australia, 39) and Clustal W Multiple Sequence Alignment software (http://workbench.sdsc.edu) (40).

Statistical Analysis

For each SNP, an assessment was performed assuming both a dominant and nondominant genetic relationship with the CBAVD phenotype, as the precise relationships between SNP genotype and TGF-β1 or EDNRA activity are not known. Differences in the distribution of SNP genotypes were compared using χ^2 analysis. In addition, a two-sample proportion test to monitor differences in overall allelic frequencies was conducted between groups. Comparisons were performed between all cases and controls collected in the study, and subdivided by ethnicity to evaluate for population-specific differences. Only historic controls were available for the Portuguese subjects; therefore, these were not included in the statistical analyses. The CFTR genotypes and polymorphism data for the Portugese group are provided in Tables 1 and 4.

Due to the selective nature of the candidate genes being explored, no corrections were made for multiple comparisons. All statistical analyses were done using SPSS (IBM; Chicago, IL) statistical software package.

RESULTS

This study was designed to pursue modifier genes contributing to the CBAVD phenotype. Eighty subjects with CBAVD (Table 1) and 51 controls were investigated for candidate polymorphisms in TGF-β1 or EDNRA. Darrah et al. (29) previously described two polymorphisms in EDNRA associated with a more severe lung phenotype among subjects with CF. Both the AA genotype of SNP rs 1801708 and the CC allele of rs 5335 were reported to occur more frequently among individuals with CF with severe lung symptoms. In a large cohort of subjects with CBAVD and controls, we observed a notable increase of the CC allele at SNP rs 5335 in association with CBAVD (Table 2). The CC allele was significantly greater in the largest matched study cohort (i.e., Turkish patients vs. controls 45.2% vs. 19.4%, $P<.05$ by χ^2 analysis), and between all cases versus controls (36% vs. 15.7%, $P<.05$). The EDNRA promoter SNP (rs 1801708) did not appear to influence the penetrance of CBAVD ($P=.22$) (for either Turkish or Spanish cases vs. controls; Table 2). Similarly, studies of the rs 1982073 (TGF-β1 codon 10) SNP indicated a trend toward increased T allelic frequency in all subjects with CBAVD compared with controls (58% vs. 45%), although neither the subgroup analyses for polymorphism distribution by ethnicity nor genotype frequency indicated a significant association with

CBAVD penetrance. With regard to TGF-β1 codon 25 SNP (rs 1800471), there was no association with CBAVD for any of the analyses performed (Tables 3 and 4).

DISCUSSION

Transforming growth factor-β is the best described modifier of the CF pulmonary phenotype. With reference to the present study, the human vas deferens, epididymis, and seminal vesicle develop from the Wolffian ducts, and it is well established that TGF-β and related signaling pathways are crucial during normal Wolffian duct development and differentiation (41–43). A rat gene expression array suggested that during Wolffian duct formation, androgens indirectly modify insulin-like growth factor (IGF) and TGF-β signaling pathways, both of which play an important role during epithelial–mesenchymal interactions and normal development of the vas (43). Although TGF-β and associated signaling pathways have been shown to subserve a crucial role in the normal vas, and clearly contribute to CFTR-related pathology in tissues such as lung, the significance of this pathway in atypical CF-related conditions, such as CBAVD, has not been studied previously.

In our experiments, we found that TGFB polymorphisms rs 1800471 and 1982073 do not impact the CBAVD clinical phenotype. The result suggests important differences in the pathogenesis attributable to altered CFTR expression in CBAVD versus pulmonary CF. For example, CF lung manifestations including polymorphonuclear cell infiltration and cytokine release are known to exacerbate CF lung injury, and TGF-β (a known inflammatory modulator) might influence the extent of pulmonary inflammation due to chronic infection. Such mechanisms may not be relevant to vas development in utero, and therefore cannot be invoked to account for CBAVD in the setting of CFTR deficiency.

On the other hand, results from this initial survey indicate that at least one known genetic modifier of CF lung disease (EDNRA) does appear to associate with CBAVD. Endothelin receptor type A or a close homologue have been implicated previously as important during normal formation of the mammalian nervous system, the anorectum, and craniofacial structures such as the mandible (44–47). Our results point to EDNRA as playing a significant role during development of the vas deferens, and indicate that the gene product may contribute to loss of the vas in the setting of CFTR insufficiency. Having said this, CBAVD is likely a multifactorial disease, and a number of other modifying factors almost certainly influence disease penetrance. Further studies of larger patient cohorts, as well as genome-wide association analyses will be necessary to determine the major effectors that influence penetrance of CBAVD.

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