Potential role of cystic fibrosis genetic modifier factors in congenital bilateral absence of the vas deferens

PhD Theses

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Introduction

Congenital bilateral absence of the vas deferens (CBAVD) is a rare condition associated with normal spermatogenesis and obstructive azoospermia due to lack of ducts that connect the epididymis to the urethra. CBAVD is usually discovered at adulthood during medical investigations for causes of clinical infertility in otherwise asymptomatic males. The etiology of CBAVD is not fully understood; however a well-established connection between CBAVD and cystic fibrosis (CF) exists. CF is the most common life-shortening autosomal recessive disorder among Caucasians, and is characterized by recurrent pulmonary infections, elevated sweat chloride, pancreatic failure, hepatic insufficiency, and other glandular defects. App. 98% of CF males is infertile and exhibits CBAVD. Cystic fibrosis is caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (*CFTR*) gene that encodes an epithelial chloride-bicarbonate channel. *CFTR* mutations have also been implicated in a variety of pathologic conditions such as disseminated bronchiectasis, allergic bronchopulmonary aspergillosis, acute recurrent or chronic idiopathic pancreatitis, alcoholic chronic pancreatitis, and (as above) CBAVD. A small subset of males with CBAVD exhibits the disease without known *CFTR* defects. However, 80-97% of CBAVD subjects possess

at least one defective *CFTR* allele, and 50-93% of individuals with CBAVD carry two variants. The F508del *CFTR* mutation (deletion of phenylalanine at amino acid position 508) is the most frequent *CFTR* mutation, app. 70% of patient carry at least one copy of this mutation. Another *CFTR* mutation at the same 508 residue - phenylalanine-to-cysteine replacement - has also been described, and been considered as a clinically silent polymorphism that does not contribute to CF or CBAVD. The F508del mutation decreases efficiency of protein folding and results in premature CFTR degradation, while F508C (rs74571530 previously rs1800093) is permissive for CFTR maturation. One in 29 Caucasian male in the US carries one *CFTR* variant but does not develop CBAVD. Other genetic factors must modify penetrance of CBAVD, however these are not yet known. Tissue growth factor β-1 (TGFβ-1) is a multifunctional cytokine that also contributes to the pathogenesis of lung fibrosis and asthma. Drumm *et al.* showed that the TGFB-1 codon 10 CC genotype (rs1800470 previously rs1982073) is associated with severe lung disease among individuals homozygous for *CFTR* mutations. A second TGFB-1 SNP in codon 25 (rs1800471) has been implicated as a contributor to CF lung disease progression in some but not all studies. In addition to these, Darrah *et al.* found that endothelin receptor type A

(*EDNRA*) genotype AA at position -231 from AUG (rs1801708) and genotype CC in exon 8 (rs5335) were associated with more severe lung disease in CF patients. Besides the well-established connection between *CFTR* mutations and CBAVD, the role of additional genetic factors behind this disorder has not been studied extensively before. Moreover, because of the extremely large number of *CFTR* sequence alterations (1,900 published so far in the CF Mutation Database), the role of most defects in CBAVD has not been determined thus far. Therefore, in our studies, we have investigated the potential role of five genetic factors - *CFTR*-F508C, TGFB-1 codon 10 and 25 SNPs, *EDNRA* promoter and exon 8 SNPs – in the pathogenesis of CBAVD.

Objectives

The central hypotheses in these projects are 1) a mild CFTR mutation (F508C) can contribute to the CBAVD phenotype and 2) cystic fibrosis genetic modifiers influence not only the disease expression of CF but also impact upon the manifestation of a CFTR-related disease: CBAVD.

<u>**Aim 1.**</u> Investigate the incidence and study the potential pathogenic role of F508C in large CF carrier, cystic fibrosis and CBAVD cohorts.

Aim 2. Examine frequencies of TGFB-1 polymorphisms (rs1800470 and rs1800471) and *EDNRA* polymorphisms (rs1801708 and rs5335) in CBAVD subjects.

Aim 3. Study the hypothesis that CF modifier genes influence CBAVD penetrance.

Materials and Methods

Subjects in our first CBAVD study included men who had undergone full *CFTR* gene sequence analysis with the Ambry Test[TM]: CF at Ambry Genetics between January 2002 and June 2007. This group was comprised of 6,970 male patients, 850 of whom were analyzed for CF carrier screening; 5,938 were submitted for diagnostic testing on suspicion of CF or with a clinical diagnosis of CBAVD (n=182). Each individual tested had given written consent to have a DNA sample available for research studies. Genomic DNA was isolated from blood leukocytes and DNA was assessed for quality and quantity by agarose gel electrophoresis. The Ambry Test[TM:]CF evaluates the *CFTR* gene by modified temporal temperature gradient electrophoresis analysis (mTTGE) followed by dye terminator DNA sequencing of suspect regions. The test covers all exons, at least 20 bases 5' and 3' into each intervening sequence, and select deep intronic mutations. Briefly, all exons as well as relevant intronic regions were amplified using polymerase chain reaction

(PCR). Prior to gel analysis, PCR products were denatured and slowly cooled to allow for maximal heteroduplex formation. For a subset of *CFTR* regions, DNA was mixed with known wild-type DNA samples to facilitate detection of homozygous mutations. PCR products were then processed for mTTGE on DCode gels. Polyacrylamide gels were analyzed for the presence of mutations following staining in ethidium-bromide and image capture under UV light using a GelDoc system. Gel analysis fragments were scored against known controls. Regions indicating the presence of a mutation by mTTGE were processed for dye terminator sequencing. Standard dye terminator cycle sequencing was followed by analysis on a CEQ8000 capillary electrophoresis sequencer.

In our second CBAVD study, we analyzed genomic DNA samples from 80 CBAVD individuals and 51 healthy male control subjects from Europe. This included 19 patient samples and 20 controls from Spain, 31 CBAVD subjects and 31 controls from Turkey, and 30 individuals with CBAVD from Portugal. 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. The protocol was approved by the Institutional Review Board of Human Use at the University of Alabama at

Birmingham and by local Portuguese, Spanish, and Turkish ethical committees. All subjects have given permission to have his DNA sample available for research studies. A 453 bp region of the 5' end of TGFB-1 gene (NT_011109) was amplified using 5'GAGGACCTCAGCTTTCCCTC3' (forward) and 5'CTCCTTGGCGTAGTAGTCGG3' (reverse) primers. This region includes both rs1800470 and rs1800471 TGFB-1 SNPs. For the amplification of the *EDNRA* gene promoter region (Ensembl Gene ID: ENSG00000151617, including SNP rs1801708) the following primers 5'GTGGAAGGTCTGGAGCTTTG3' and 5'TTCCCAGCTCTCGTCTTCTC3' were used. For the detection of the exon 8 SNP of the *EDNRA* gene (rs5335), we used primers: 5'CTGCTGCTGTTACCAGTCCA3' and 5'TGACCAGTTCCCATTGAACA3'. QIAquick PCR Purification Kit was used prior to sequence analyses. The sequencing products were run according to standard protocols on an Applied Biosystems 3730 Genetic Analyzer with POP-7 polymer. Sequence analyses and comparisons were conducted using Clustal W Multiple Sequence Alignment and Chromas Lite softwares.

A 3-way Chi-Square contingency test was performed to ascertain the significance of F508C frequency differences among the three study populations. Statistical analyses were

performed using the VassarStats website. Since F508C was present in fewer than 5 subjects in two of the three study groups, a Monte Carlo simulation of a three categorical sampling distribution was run for an additional 10,000 samples. Pairwise comparisons between the subdivided groups, as well as odds ratios (OR), were also calculated.

For each TGFB-1 and *EDNRA* SNP, an assessment was performed assuming both a dominant and non-dominant genetic relationship with the CBAVD phenotype, as the precise relationships between the SNP genotype and TGFB-1 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; and subdivided by ethnicity to evaluate for population specific differences. 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 statistical software package.

Results

Genotype data for F508C was obtained from 850 male subjects undergoing complete *CFTR* sequence analysis for CF

carrier screening, as well as a group of 182 subjects with a clinical diagnosis of CBAVD. In addition, data from 5,938 patients submitted for diagnostic testing on suspicion of CF were also analyzed. Among the 850 individuals referred for CF carrier screening, 3 (0.35%) subjects had the F508C variant without a second mutation. When *CFTR* from 182 CBAVD individuals was analyzed, a total of 3 (1.65%) individuals were identified as carriers of F508C in addition to another mutation (Table 1.). In 18 specimens submitted for diagnostic testing on suspicion of CF, F508C was the only detected variant. In 5 CF individuals, the F508C variant was present along with a second mutation (Table 2.). Comparatively, the allelic frequency of F508C in the CBAVD population was 4.68 times higher than the CF carrier screening population. Detailed F508C frequency data from all three study groups are presented in Table 3. below. When comparing allelic frequencies of F508C in the group of patients with CBAVD to those CF patients for whom F508C was observed with a second *CFTR* mutation, F508C among CBAVD patients was 19.6 times more frequent. Two-way χ^2 tests revealed that the frequency of F508C was significantly higher in the CBAVD group than any other group (Table 3.). Comparison of the F508C frequencies between the CF carrier screen group and the group submitted for diagnostic testing

on suspicion of CF showed no detectable difference (χ^2=0; p=1).

Table 1. Genotypes of CBAVD patients with F508C.

Patient #	Mutation 1	Mutation 2	Clinical History	Age
1	F508del	F508C	CBAVD	39
2	F508del	F508C	CBAVD, sinusitis, asthma	34
3	L206W	F508C	CBAVD	40

Table 2. Genotypes of patients with F508C submitted for diagnostic testing on suspicion of CF.

Patient #	Mutation 1	Mutation 2	Clinical History	Age
1	G551D	F508C	Positive newborn screen	>1 mo
2	V754M	F508C	Clinical suspicion of CF	1 mo
3	DeltaF508	F508C	Clinical suspicion of CF	24 yrs
4	DeltaF508	F508C	Clinical suspicion of CF	11 yrs
5	DeltaF508	F508C	Clinical suspicion of CF	32 yrs

In a more robust three-way comparison, the frequency of F508C in individuals with CBAVD was significantly increased relative to both the CF carrier screen group and the group submitted for diagnostic testing on suspicion of CF (χ^2=6.95; p=0.031). The Monte Carlo simulation for 10,000 replications yielded a cumulative probability of 0.0486.

Table 3. F508C frequencies in all study groups

	Patients with CBAVD (F508C plus another mutation)	CF carrier screen (F508C only)	Patients submitted for diagnostic testing on suspicion of having CF		Total
			F508C plus another mutation	F508C only	
Patients with F508C	3	3	5 [b]	18	29
Total patients *Alleles*	182 *364*	850 *1,700*	5,938 *11,876*	5,938 *11,876*	6,970 *13,940*
F508C allele frequency	**0.824%**	0.176%	0.042% 0.151% 0.193%		0.208%
χ^2, (p)		2.38 (0.073)	21.94 **(0.002)** 5.87 **(0.023)** 4.01 **(0.038)**		
Odds ratio		**4.701**	**4.283**		

Chi square and odds ratios are shown for the group with a diagnosis of CBAVD compared to the CF carrier screening, patients submitted for diagnostic testing on suspicion of CF, or all other pooled groups in pairwise comparisons. P values shown are corrected for 10,000 samples simulation.
[b] Determined to carry one disease-associated *CFTR* mutation in addition to F508C.

In our modifier gene study, we tested whether codon 10 and codon 25 TGFB-1 polymorphisms, or either of the two *EDNRA* gene polymorphisms might act as genetic modifiers of CBAVD. The *EDNRA* exon 8 CC allele was significantly more frequent in the largest matched study cohort (i.e. Turkish patients vs. controls 45.2% vs. 19.4%, $p<0.05$ by χ^2-analysis), and between all cases vs. controls (36% vs. 15.7%, $p<0.05$). The rs1801708 SNP did not appear to influence the penetrance of CBAVD ($p=0.22$) for either Turkish or Spanish cases vs. controls (Table 4).

Table 4. EDNRA genotype distributions in CBAVD patients and controls

EDNRA polymorphisms			Patients		Controls	
Exon 8 (rs 5335)	*Turkish*	CC [d]	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 [e]		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	

[d] $p < 0.05$ for Turkish population, χ^2 analysis
[e] $p < 0.05$ for all subjects shown, χ^2 analysis

Studies of the rs1800470 SNP indicated a trend towards increased T allelic frequency in all CBAVD subjects compared to controls (55% vs. 45%), although none of the subgroup analyses indicated a significant association with CBAVD penetrance. With regard to TGFB-1 codon 25 SNP (rs1800471), there was no association with CBAVD for any of the analyses performed. We observed a notable increase of the CC allele at SNP rs 5335 in association with CBAVD (shown in Table 4). Detailed TGFB-1 genotype distribution data are listed in Table 5.

Table 5. **TGFB-1 genotype distribution in CBAVD patients and controls**

TGFB-1 polymorphisms			Patients		Controls	
Codon 10 (rs1800470 previously rs1982073)	*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 (rs1800471)	*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	

Conclusions

- The F508C-*CFTR* mutation frequency was significantly elevated among subjects with CBAVD compared to either CF carriers or CF patients. Based on our results, F508C is causative for both CBAVD and CF when present with a second *CFTR* mutation. However, because of the significantly elevated frequency of the mutation among CBAVD subjects, we can conclude that F508C (together with an additional *CFTR* gene defect) is more likely to lead to CBAVD than CF.

- TGFB-1 polymorphisms rs 1800470 and rs 1800471 do not affect CBAVD penetrance. Thus, TGFB-1 - a known genetic modifier of cystic fibrosis lung disease – did not influence CBAVD in our subject groups.

- The *EDNRA* exon 8 CC allele was significantly more prevalent among CBAVD patients and controls from Turkey and between all cases vs. controls. The *EDNRA* promoter SNP (rs1801708) did not influence the CBAVD phenotype in our study groups. Based on these, endothelin receptor type A gene serves as a CBAVD specific genetic modifier.

  These studies provide important new information regarding the genetic factors that contribute to CBAVD. Large, multicenter studies will be needed to clarify the role of other, classically non-disease-associated *CFTR* mutations and putative modifier genes in CBAVD.

Publications list

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