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Aetiology of congenital absence of vas deferens: genetic study of three generations

Pasquale Patrizio[1,4], Ricardo H.Asch[1], Barbara Handelin[2] and Sherman J.Silber[3]

[1]University of California, Irvine, Division of Reproductive Endocrinology and Infertility, Orange, CA, [2]Integrated Genetics, Framingham, MA and [3]St. Luke's Hospital, St. Louis, MO, USA

[4]To whom correspondence should be addressed at: University of California, Irvine, Division of Reproductive Endocrinology, 101 The City Drive, Pavilion II, Orange, CA 92668, USA

Bilateral congenital absence of the vas deferens (CAVD) is a form of male sterility (found in otherwise normal men) of unknown aetiology. Because males with cystic fibrosis (CF) almost invariably have CAVD as well, we investigated the hypothesis that men with isolated CAVD might share a common genetic background with males with CF. Genetic testing for CF was carried out in three generations of subjects: 44 patients with CAVD and their wives, 24 of their parents, and 13 of their offspring generated by microsurgical epididymal sperm aspiration (MESA) and in-vitro fertilization (IVF). DNA extracted from peripheral lymphocytes was amplified by the polymerase chain reaction (PCR) and then analysed for 12 mutations in the cystic fibrosis transmembrane conductance regulatory (CFTR) gene. Among 44 patients tested with CAVD, 26 (59%) were positive for at least one CF mutation, while the carrier frequency for CF mutations in the general population is only 4%. Four patients were found to be compound heterozygotes, three with genotypes Delta F-508/R117H, one with R553X/R117H. Among 24 parents tested, 15 (seven fathers, eight mothers) had sons with CAVD who were positive for CF mutations. Of these, nine (four fathers and five mothers) were found to be carriers for CF mutations. These four fathers, although carriers of CF mutations, were obviously fertile. Of the 13 offspring tested, six (three boys and three girls) had CF positive fathers. Of these, three (two girls and one boy) were found to be carriers for CF mutations. These MESA/IVF children are the first offpsring to whom men with CAVD have been able to transmit CF mutations. All of the MESA/IVF male offspring (like their grandfathers) had a normal vas deferens bilaterally, including one carrier for Delta F-508. This study revealed, by genetic testing of otherwise normal men with sterility caused by CAVD, a new population of patients with a variant form of CF and highlighted the possibility that carrier frequency for CF is higher than previously thought. Compound heterozygosity for CF mutations and not carrier condition is associated with isolated CAVD. It is concluded that genetic counselling and screening for CF should be offered to couples undergoing sperm aspiration and IVF procedures when CAVD is a factor in their infertility.

Key words: epididymal sperm/cystic fibrosis/genetics/male infertility

Introduction

Bilateral congenital absence of the vas deferens (CAVD) with obstructive azoospermia is a reproductive disorder of which the aetiology has for a long time been unknown (Michaelson, 1949; Charny and Gillenwater, 1965). It represents 1.5% of all cases of male infertility and ~25% of all cases of obstructive azoospermia (Jequier *et al.*, 1985; Dubin and Amelar, 1971; Patrizio and Asch, 1990). In the USA alone it is estimated that ~35 000 to 40 000 men are azoospermic because of vas deferens agenesis (Asch and Silber, 1991). This particular type of male infertility was always considered untreatable until 5 years ago when a new technique, consisting of microsurgical epididymal sperm aspiration combined with in-vitro fertilization (MESA/IVF) and tubal embryo transfer (TET), was successfully used (Silber *et al.*, 1987, 1990; Patrizio *et al.*, 1988).

Infertility caused by CAVD is also a common finding in males with cystic fibrosis (CF) disease (Kaplan *et al.*, 1968; Holsclaw *et al.*, 1971; Taussig *et al.*, 1972; Seale *et al.*, 1985). CF is the most common autosomal recessive disorder in North American Caucasians, with an estimated disease frequency of 1:2500 live births and a carrier frequency of 1:25 (Boat *et al.*, 1989; Christian *et al.*, 1990). Recently, the defective gene responsible for CF has been identified on the long arm of chromosome 7 and cloned (Rommens *et al.*, 1989; Riordan *et al.*, 1989). This gene, designated CFTR (cystic fibrosis transmembrane conductance regulator), is predicted to code for a transmembrane protein of 1480 amino acids which appears to regulate the transmembrane transport of ions (most likely Cl^-) into epithelial cells (Quinton, 1983; Widdicombe *et al.*, 1985). Holsclaw *et al.* (1971) hypothesized that the association of azoospermia with CAVD could represent an incomplete form of CF disease. However, no genetic tests to verify such a hypothesis have been available until recently, when the gene for CF disease was identified and cloned.

The striking observation that men with CF are infertile because they have no vas deferens, along with the identification of the gene for CF and with the availability of assays for detecting many of its different mutations, has led us to investigate the hypothesis that the infertile population of men with isolated CAVD might share a common genetic background with CF patients, representing perhaps mild or incomplete forms of the same disease.

Recent studies have suggested an increased frequency of the common CF mutation (Delta F-508) (Dumur *et al.*, 1990; Rigot *et al.*, 1991) or some of the rarer ones (Anguiano *et al.*, 1992) in patients with CAVD.

Of particular interest in this study is the performance of genetic testing for CF on three generations of subjects, represented by patients with CAVD, their parents and their offspring generated with the aid of assisted reproductive technology.

Materials and methods

Subjects

A total of 44 unselected white infertile men with isolated bilateral CAVD, 28 wives, 24 of their parents (13 mothers and 11 fathers) and 13 offspring (of which two were sets of dizygotic twins) were included in this study. All the 44 men were patients in our MESA/IVF programme. The mean age was 33.5 ± 5.4 years (range 23–49). The diagnosis of CAVD was originally made on physical examination and then was confirmed at the time of the surgical sperm aspiration. The details and technical aspects of the procedure have been reported elsewhere (Silber *et al.*, 1990). None of the patients had pulmonary or gastro-intestinal signs or symptoms of CF, therefore no sweat chloride tests were performed routinely. Chest roentgrams were normal and none had symptoms of malabsorption. None had relatives or siblings with CF disease. Two patients were a set of monozygotic twins. Most were of Northern European ancestry, 11 were Jewish, one Arabic and two Italian.

Technique for genotyping

Peripheral blood samples were used to extract genomic DNA from buffy coats collected in sodium citrate using high salt precipitation of proteins. DNA samples were then amplified by the polymerase chain reaction (PCR) and resulting products were analysed for 12 mutations in the CFTR gene. Exon 4,10,11,20 and 21 were amplified in a multiplex reaction followed by allele specific oligonucleotide (ASO) probe analysis for the mutations Delta F508, G542X, G551D, R553X, W1282X, N1303K, Delta I507, 1717G-A, R560T, S549N, R117H and 621+1.

Primer sequences for each of the PCR reactions were as described elsewhere (Kerem *et al.*, 1989a, 1990; Cutting *et al.*, 1990; Dean *et al.*, 1990). Briefly, 1 μg of genomic DNA was mixed with 10 μM of each PCR primer and amplified in a Perkin Elmer System 9600 for 36 cycles of 10 s at 94°C, 10 s at 55°C and 30 s at 74°C. Five separate genomic regions within the CFTR gene were amplified simultaneously in a single amplification reaction. Amplified samples were spotted onto nylon membrane (ICN, Biotrans) under high salt denaturing conditions and then hybridized with 17-mer ASOs under 3 M tetramethylammonium chloride (TMAC) conditions as previously described (Kerem *et al.*, 1989; Wood *et al.*, 1985). ASOs were 5'-end labelled with [gamma-^{32}P]ATP (NEN) at 6000 Ci/mmol and T4 polynucleotide kinase (NEB) according to the manufacturer's recommendations. Following hybridization, filters were washed once in TMAC buffer at room temperature followed by single wash at 52°C. Filters were blotted dry, covered with clingfilm and exposed to Kodak XAR-5 film at 70°C using intensifying screens (Dupont/NEN, Billerica, MA, USA).

Results

Of the 44 patients with isolated CAVD who were genetically screened for 12 mutations in the CFTR gene, 26 (59%) tested positive for at least one CF mutation, while the carrier frequency for CF in the general population is known to be 4% (Boat *et al.*, 1989). The details of the genotype screening for the 26 positive patients, their wives, parents and offspring are summarized in Table I.

In 13 cases the mutation identified was Delta F508. This is the most common CF mutation, a three pair base deletion, that occurs on exon 10 causing loss of a phenylalanine residue in the CFTR protein. In six patients the mutation identified was W1282X, a nonsense mutation, localized on exon 20 and predicting the production of truncated polypeptides of CFTR: one patient was found to have the R553X mutation, a nonsense mutation of the CG to TG rule localized on exon 11; one patient had the mutation 1717G-A, localized on intron 10, which would cause defective RNA splicing; one patient was found to have the G542X mutation, a nonsense mutation in exon 11. Four of the 26 patients were found to be compound heterozygotes, three with genotypes Delta F508/R117H and one with R553X/R117H. The mutation R117H was a missense mutation localized on exon 4.

The frequency distribution of the CF mutations among all copies of chromosome 7 (n = 52) of these 26 patients was markedly different from that seen in typical CF patients. For example, the frequency of Delta F508 was only 31% among CAVD patients compared to 73% among CF patients, and two

Table I. Details of the cystic fibrosis (CF) mutations identified in patients with isolated bilateral congenital absence of the vas deferens (CAVD) and their families

Patient no.	Patient's allele	Paternal allele	Maternal allele	Wife's allele	Offspring's allele
1.	DF508/	Neg.	DF508	Neg.	(g) DF508
2.	DF508/	NA	Neg.	Neg.	—
3.	DF508/	NA	NA	NA	—
4.	DF508/	NA	NA	NA	—
5.	DF508/	NA	NA	NA	—
6.	DF508/	DF508	Neg.	Neg.	(g) Neg.
7.	DF508/	NA	NA	NA	—
8.	DF508/	NA	NA	Neg.	—
9.	DF508/	NA	NA	Neg.	—
10.	DF508/	Neg.	DF508	Neg.	—
11.	DF508/	NA	NA	Neg.	—
12.	DF508/	NA	NA	Neg.	—
13.	DF508/	NA	NA	Neg.	—
14.	W1282X/	NA	NA	NA	(b) Neg.
16.	W1282X/	Neg.	W1282X	Neg.	—
17.	W1282X/	NA	NA	NA	—
18.	W1282X/	NA	NA	Neg.	—
19.	W1282X/	NA	NA	Neg.	—
20.	DF508/R117H	DF508	R117H	Neg.	(g) DF508
21.	DF508/R117H	NA	NA	NA	(b) DF508
22.	DF508/R117H	R117H	DF508	R117H	—
23.	R553X/R117H	NA	NA	NA	—
24.	R553X/	NA	NA	NA	—
25.	1717G-A/	1717G-A	Neg.	NA	(b) Neg.
26.	G542X/	NA	NA	Neg.	—

b = boy; g = girl; NA = data not available; —, no offspring; DF508 = Delta F508.

of the less common CF mutations were seen in significantly greater frequency in CAVD patients: W1282X had a frequency of 12% compared to <2% among CF patients, and R117H had a frequency of 8% compared to <1% in CF patients. All the percentages were based on a mixed North American Caucasian population of ~800 CF chromosomes summarized in Table II.

Out of the 24 parents tested, 15 (seven fathers and eight mothers) had sons with CAVD sterility as well as being CFTR mutation positive (CAVD-CF+), while nine (four fathers and five mothers) had CAVD sons in whom no CF mutation was detected (CAVD-CF−). Table I shows that of the eight mothers tested in the CAVD-CF+ group of patients, five were found to be carriers (one for R117H, one for W1282X, and three for Delta F508). Of the seven fathers whose sons were CAVD-CF+, four were found to carry a mutation (two for Delta F508, one for 1717G-A and one for R117H). However, these fathers, despite their CF carrier status, were obviously fertile and able to father their own progeny. These results indicated an autosomal recessive inheritance of CAVD and ruled out the possibility that, in patients with CAVD whose parents tested positive for CF, new mutations appeared. All of the nine parents whose sons were CAVD-CF− also tested negative to the CF screening.

We were specifically interested in ascertaining the mode of inheritance in the four patients who were found to be compound heterozygotes (Table III). To date, we have obtained blood samples on two sets of parents and found that each parent was a carrier for a CF mutation transmitted to their son; each parent must also have contributed to the CAVD phenotype.

Finally, we tested the fidelity of transmission of the CF mutations from CAVD patients to their offspring who resulted from MESA/IVF and TET. Of the 13 offspring from whom blood samples could be obtained, six (three boys and three girls) had fathers in whom CF mutations were detected. Of these six children, three (two girls and one boy) were found to be carriers for the Delta F-508 mutation. None of the seven children born to CF negative fathers tested positive.

These MESA/IVF children are the first offspring to whom men with CAVD have been able to transmit CF mutations. However, none of the male offspring who were tested, including the one carrier for Delta F508, inherited CAVD. On physical examination of these male offspring, in fact, the presence of a normal bilateral vas deferens was demonstrated in all.

Of the 28 wives tested, only one (3.6%) was found to be a carrier for a CF mutation (R117H), while her husband was a compound heterozygote (Delta F508/R117H). In this couple, despite successful IVF/ET, no offspring have been born.

Table II. Cystic fibrosis (CF) mutation frequency in patients with CF and isolated bilateral congenital absence of vas deferens (CAVD)

CF mutation	Frequency in CF patients	Frequency in CAVD CF positive patients
R117H	0.3% (3/812)[a]	8% (4/52)
W1282X	1.6% (9/578)	12% (6/52)
DF508	73% (593/812)	31% (16/52)

[a](3/812) indicates three R117H alleles among 812 CF alleles (in 406 patients).

Table III. Allele distribution for three generations in patients found to be compound heterozygotes

No.	Patient allele	Paternal allele	Maternal allele	Wife allele	Offspring	
					Allele	Phenotype
1.	R553X/R117H	NA	NA	NA	—	—
2.	DF508/R117H[a]	DF508	R117H	Neg.	(g) DF508	Normal
3.	DF508/R117H	NA	NA	NA	(b) DF508	Normal (− vas)
4.	DF508/R117H[a]	R117H	DF508	R117H	—	???

b = boy; g = girl; NA = data not available.
[a]Normal sweat chloride tests.

Discussion

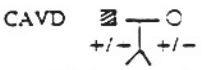
This study provided information on the genotype of three consecutive generations of subjects in order to elucidate the aetiology of isolated CAVD and its relationship to CF. We analysed a total of 12 CF mutations, these 12 accounting for >87% detection of known mutations in a classical CF population (Ng *et al.*, 1991). Our population included 44 patients with CAVD and 28 of their wives, 24 of their parents and 13 of their offspring. The number of parents available for genetic testing was limited for reasons such as residing at a great distance, or

Table IV. Probability for an individual to be a carrier and for a child to be affected with cystic fibrosis (CF)

	Risk to be a carrier	Risk not to be a carrier
Prior	1/25	24/25
Conditional (given test results)	0.15	1
Joint	0.006	0.96
Posterior	0.006	

0.006 ÷ 0.96 = 0.0062 or 0.6%

Then, the risk that the couple would have a child with CF is:



(1) (0.006) (0.5) = 0.3% probability to have child with CF

+/+ = presence of CF mutation, +/− = 0.6% probability to be carrier; +/+ has probability 1 of passing CF mutation to child, +/− has probability 0.5 +/− of passing CF mutation to child.

because the patients preferred not to involve them due to the privacy of their condition, or because they were no longer alive.

Twenty-six patients with isolated CAVD (59%) were found positive for at least one CF mutation, in contrast to the 4% incidence of CF carriers in the general population (Boat *et al.*, 1989). Of these, four patients were found to be compound heterozygotes, three with genotype R117H/Delta F508 and one with R117H/R553X. Since these genotypes are also found in patients with mild CF disease (Dean *et al.*, 1990), it is tempting to speculate that CAVD is a genetically transmitted condition closely related to CF, representing perhaps a mild or incomplete form of the same disease. Our results confirm those in a recent report by Anguiano *et al.* (1992), which described an incidence of 64% detectable CF mutations in 25 patients with CAVD. However in the present study, we extended the genetic screening to the parents and the offspring of men with CAVD and moreover, by testing for 12 CF mutations, we demonstrated other rare CF mutations (R117H, G542X and 1717G-A) in patients with CAVD.

Since the prevalence of single, identifiable CF mutations among CAVD patients is high, it might be thought that CF carrier status alone is sufficient to cause CAVD (Dumur *et al.*, 1990; Oates *et al.*, 1991; Rigot *et al.*, 1991; Silber *et al.*, 1991). However, by analysing the results of genetic testing of fathers of patients with CAVD, we found four carriers for known CFTR mutations of which two carried Delta F508, one carried R117H and one 1717G-A. These fathers, despite their carrier condition, had a normal vas deferens on physical examination and had no problem with their reproductive function. Likewise, all the male progeny originating from patients with CAVD from the MESA/IVF technique had a vas deferens bilaterally, clearly appreciated on physical examination, and including the male offspring found to be a carrier for Delta F508.

Therefore, men with isolated CAVD identified as carriers for CF, are in reality not 'true carriers' like their fathers or offspring. Most likely they are compound heterozygotes, with the mutation on the other member of the chromosome pair either not yet identified or simply not included in the panel of 12 CFTR mutations assayed in the present study. Support for this hypothesis comes from data recently published where additional rare mutations for CF were identified in a population of men with CAVD who previously were found to be only carriers of more common CF mutations (Anguiano *et al.*, 1992). Similarly, the 18 patients with CAVD found negative to the CF testing in this study, could carry two rare mutations yet to be identified.

The data obtained so far from parental genetic analysis of patients with CAVD also indicate that there is no particular CF allele, whether on the maternal or on the paternal side, which is predictive for the absence of vas deferens in their sons.

In the four patients identified as compound heterozygotes for two CF mutations and whose only CF-related symptom is absence of the vas deferens, all have one R117H mutation in combination with either Delta F508 ($n = 3$) or R553X mutations ($n = 1$). Sweat chloride tests were only performed on two of the genotypes Delta F508/R117H and both were normal (40 and 45 mmol/l). This is the first time that compound heterozygotes for Delta F508/R117H have been found to have normal sweat chloride tests and CAVD as the only phenotypical expression of CF disease. This finding is consistent with another report of mild CF cases involving Delta F508/R117H genotypes (Dean *et al.* 1990) where it appears that the R117H mutation opposite Delta F508 may moderate the more severe phenotype of the homozygous Delta F508 genotype. However, since other CF patients with good pulmonary function and pancreatic sufficiency have been found to have various genotypes, including homozygous Delta F508 (Kerem *et al.*, 1990; Bonduelle *et al.*, 1991; Barreto *et al.*, 1991), it appears that genotype alone does not account for the wide variability seen in phenotype. Therefore, to explain the very mild phenotype of CAVD we suggest that there must be either modifying sequences associated with specific CFTR alleles, or that there is a second, perhaps closely affiliated gene product which modifies the activity/function of CFTR in different tissues.

The six CFTR mutations identified in this study occur on four exons plus one intron and represent deletion (Delta F508), nonsense (W1282X, 1717G-A and G542X) and amino acid substitution (R553X and R117H) mutations. R117H is one of the transmembrane regions of CFTR, Delta F508, W1282X and R553X are in nucleotide binding regions, and 1717G-A is at the splice border of intron 10 (Kerem *et al.*, 1990; Cutting *et al.*, 1990; Dean *et al.*, 1990). As such, there does not yet appear to be any unifying theme of types of mutations or locations within the protein product to explain the very distinct phenotye seen in these patients. Indeed, 1717G-A, Delta F508 and W1282X are associated with the pancreatic insufficient CF phenotype (Kerem *et al.*, 1990) which tends to be a more severe phenotype, whereas R117H has been found only in mildly affected individuals (Dean *et al.*, 1990). Four of the six patients with the W1282X mutation were Jewish Ashkenazi and this is in agreement with a previous report where W1282X was found to be the most common CF mutation in this ethnic group (Shashani *et al.*, 1992).

The hypothesis that CAVD patients are probably compound heterozygotes of known and unknown CFTR mutations would add an unknown quantity of previously unrecognized CF mutations into the gene pool, thus increasing the population carrier frequency. Indeed, if the estimates of CAVD are correct (35 000 to 40 000 men in the USA) (Asch and Silber 1991), then the incremental increase due to CAVD almost doubles the estimates of the CF carrier frequency from 1/25 to ~ 1/15. This figure does not include predicting an equivalent number of female compound heterozygotes who remained clinically undetected. Our findings also should heighten suspicion that there may be other unrecognized populations of carriers and complex heterozygotes of CFTR mutations. Men with isolated CAVD not only should provide new insights into the complex heterogeneity of the CF disease (Schwachman, 1972; Strong *et al.*, 1991) but they also represent a good population model to be studied for the identification of the remaining mutations related to CF.

In conclusion, the discovery of CFTR mutations in a patient population previously unsuspected of carrying them, namely, infertile men with isolated CAVD, leads to a new understanding of the aetiology of CF. Isolated CAVD could be defined as an incomplete or variant form of CF, inherited as an autosomal recessive condition in sons of carriers for different CF mutations.

We recommend genetic testing and counselling for each couple with CAVD sterility participating in a MESA/IVF programme, with particular emphasis on genetic testing of the wives. If the

wives are CF negative then the risk of having offspring with CF, as shown in this study, is minimal (~0.3%). The method to calculate the probability that an individual (wives in this case) could be a carrier of an unidentified CF mutation and the risk to have a child with CF are shown in Table IV. At the same time the chance of having male offspring with CAVD, although not clear because of still undetermined mutations, is also likely to be low. The counselling becomes more difficult in cases where wives are CF carriers. In such instances (case #4, Table III) we do not know if a future child will be at risk for CF or, if male, only for CAVD. This obviously presents a very difficult counselling situation since the CAVD phenotype is not only extremely mild, but its associated infertility can successfully be overcome with the MESA/IVF procedure (Silber *et al.*, 1990). As a final point, it is suggested that screening for CF mutations might become part of the clinical tests available in the initial work-up of all infertile azoospermic men to preclude the diagnosis of CAVD.

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