

# Linkage disequilibrium between the M470V variant and the IVS8 polyT alleles of the CFTR gene in CBAVD

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## Abstract

**Congenital bilateral absence of the vas deferens (CBAVD) is a cause of male sterility mostly resulting from mutations in the cystic fibrosis transmembrane regulator (CFTR) gene. The most common defect is the 5T variant at the branch/acceptor site of intron 8, which induces high levels of exon 9 skipping leading to non-functional protein. However, this 5T variant has incomplete penetrance and variable expressivity, suggesting that some other regulatory factors may modulate the splicing of exon 9. To identify such factors, we report here the genetic analysis of a polymorphic locus, M470V, located in exon 10 of the CFTR gene in 60 patients with CBAVD, compared to a normal control population. The statistical analysis showed strong linkage disequilibrium between the 5T allele and the V allele of the M470V polymorphism in the CBAVD population, but not in the normal population. The V allele in a gene carrying 5T could, however, contribute to lowering the level of normal transcripts, as already suggested by in vitro transcriptional studies. These genetic findings, together with previous studies, suggest involvement of the M470V variant in the modulation of the splicing of exon 9 of the CFTR gene.**

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Cystic fibrosis (CF) is a severe autosomal recessive disease caused by mutations in the cystic fibrosis transmembrane regulator (CFTR) gene.<sup>1-3</sup> The disease shows a highly variable clinical presentation. Genotype-phenotype correlations have been established for some mutations, although substantial variability in disease expression can still be observed among patients carrying the same mutation. Almost all CF men are infertile as a result of congenital absence of the vas deferens (CBAVD). CBAVD can also be found in otherwise healthy men. The high frequency of CFTR mutations among men with CBAVD indicates that isolated CBAVD could be an incomplete form of CF.<sup>4</sup> The 5T variant at the branch/acceptor site of intron 8 is the most common CFTR defect in CBAVD patients.<sup>5,6</sup> This site has three alleles with nine, seven, and five thymidines (9T, 7T, 5T, respectively). The 5T allele was found to induce higher levels of

exon 9 skipping compared to the 9T and 7T alleles, leading to non-functional protein.<sup>7</sup> Thus, the 5T allele is now considered as a mutation, even with partial penetrance and great variability in disease expression. The first molecular basis of partial penetrance of this splicing mutation has been published recently by Rave-Harel *et al*,<sup>8</sup> who reported great variability in the efficiency of the splicing mechanism among distinct organs of one subject and between subjects as well. They established a correlation between the level of correctly spliced RNA transcribed from the gene carrying the 5T allele and disease expression in both respiratory and epididymal epithelium. Furthermore, Teng *et al*<sup>9</sup> reported that the proportion of transcripts lacking exon 9 was increased in vas deferens cells compared with nasal epithelial cells, independently of the intron 8 Tn genotype. These results suggested that the level of normal transcripts depended on both intron 8 Tn allele and tissue, in which different regulation levels were observed, thus indicating the involvement of trans acting factors. In other respects, the exon 10 M470V locus is the most polymorphic two allele locus of the CFTR gene. In 1996, Cuppens *et al*<sup>10</sup> reported an increased proportion of mRNA lacking exon 9 when transcribed from a CFTR gene carrying the V allele. They further showed by in vitro studies that the V allele yielded a lower functional CFTR protein rate, at both transcriptional and translational levels, independently of the intron 8 Tn genotype. The aim of our study was to elucidate if the observed influence of the M470V locus on exon 9 skipping in vas deferens cells was restricted to the molecular level or if it could have any pathological consequences.

We analysed the intron 8 Tn tract<sup>5</sup> and the M470V locus<sup>11</sup> in 64 unrelated CBAVD patients comprising 24 carrying two mutations in the CFTR gene, 18 carrying one mutation and the 5T allele, seven carrying one mutation, two carrying the 5T allele, one homozygote for the 5T allele, and 12 carrying no mutation.<sup>12</sup> A total of 266 normal chromosomes from normal related subjects and parents of CF children were used as a control group. Statistical analysis was performed using GENEPOP (version 3.0) software.<sup>13</sup>

Haplotypes could be established for only 60 of the 64 patients (table 1), who were thus included in the statistical analysis. The 5T allele was found on 20 chromosomes in the non- $\Delta$ F508 group of chromosomes and was

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Table 1 Frequencies of the intron 8 (T)n-M470V haplotypes in CBAVD and normal chromosomes

	Haplotypes						Total
	9-M	9-V	7-M	7-V	5-M	5-V	
CBAVD chromosomes							
ΔF508	27	2	0	0	0	0	29
Non-ΔF508	10	3	27	31	4	16	91
Total	37	5	27	31	4	16	120
Normal chromosomes	16	7	85	147	7	4	266

not found associated in cis with an identified mutation. The aim of the statistical analysis of the genotypic results was to detect if the genotypic distribution was significantly different between CBAVD and normal populations. Analysis of allele frequencies at both the Tn and M470V loci showed a non-identical allelic distribution in CBAVD and normal populations (Fisher's exact test,  $p < 10^{-4}$  and  $p = 0.004$  respectively). Since 29/120 (24%) CBAVD chromosomes carried the ΔF508 mutation, which is in complete linkage disequilibrium with 9T and M alleles, a subgroup of CBAVD chromosomes that did not carry the ΔF508 mutation (CBAVD ΔF-) was analysed in the same way. The distribution of Tn locus alleles remained significantly different from the normal group (Fisher's exact test,  $p < 10^{-4}$ ). By contrast, frequencies of M and V alleles were not different from the control group (Fisher's exact test,  $p = 0.46$ ), showing that the difference previously observed in the allelic distribution at the M470V locus was mostly the result of the high frequency of the ΔF508 mutation in the CBAVD group. To analyse the linkage disequilibrium hypothesis against independence for both the Tn and M470V loci, we tested the three groups of chromosomes (CBAVD, CBAVD ΔF-, normal). We found linkage disequilibrium in the three populations (Fisher's exact test, CBAVD:  $p < 10^{-4}$ , CBAVD ΔF-:  $p = 0.00015$ , normal:  $p = 0.0023$ ). Furthermore, we performed pairwise linkage disequilibrium analysis using the Linkdos program in GENEPOP<sup>14</sup> in order to measure the effect for each allele (table 2). Positive linkage disequilibrium was detected between 9T and M alleles in the three populations and between 7T and V alleles in the CBAVD and the control groups; it was not significant in the CBAVD ΔF- group. The most striking result concerned the 5T allele. In contrast with the normal population in which no significant association was found between 5T and M470V alleles, the 5T allele was found to be strongly associated with the V allele in both groups of CBAVD chromosomes.

Table 2 Analysis of linkage disequilibrium between Tn and M470 locus in the CBAVD, CBAVD, ΔF508-, and normal groups with Linkdos program

Alleles	CBAVD		CBAVD ΔF508-		Normal	
	LD	p value	LD	p value	LD	p value
9T-M	(+)	0.0001	(+)	0.0117	(+)	0.0030
9T-V	(-)	0.0001	(-)	0.0117	(-)	0.0030
7T-M	(-)	0.0292	(0)	0.7005	(-)	0.0006
7T-V	(+)	0.0292	(0)	0.7005	(+)	0.0006
5T-M	(-)	0.0003	(-)	0.0099	(0)	0.1107
5T-V	(+)	0.0003	(+)	0.0099	(0)	0.1107

LD=means linkage disequilibrium. (+) Association and (-) lack of association between alleles, (0) indicates absence of LD.

This positive linkage disequilibrium between 5T and V alleles in a CBAVD population has not been reported previously and suggests that the M470V polymorphism could contribute to the variable expressivity of the 5T allele.

The V allele cannot be considered as a disease predisposing factor by itself since its frequency is not increased in the CBAVD population. Observation of linkage disequilibrium between the 5T and V alleles in the CBAVD population suggests additional effects of both alleles in the severity of the phenotypic expression. Such genetic results are in complete agreement with those obtained at the molecular level. Indeed, it has been shown that (1) the disease expression was correlated with the normal transcript level; CBAVD patients thus have low levels of correctly spliced RNA in the vas deferens, and (2) the rate of exon 9 skipping depended both on Tn tract<sup>7</sup> and M470V alleles<sup>10</sup> and on tissue.<sup>8,9</sup> The V allele in a 5T carrying gene should thus aggravate the effect of the 5T allele on the splicing mechanism defect and contribute to even lower levels of normal transcripts. In the study of Rave-Harel *et al.*,<sup>8</sup> however, the level of normal CFTR transcripts was no different between the M and V alleles in the respiratory epithelium, but no data concerning the epididymal epithelium were shown. As the studies of both Teng *et al.*<sup>9</sup> and Rave-Harel *et al.*<sup>8</sup> strongly suggest different regulation levels in both tissues, the observation of an equivalent rate of normal transcripts for the M and V alleles in respiratory tissue does not apply equally to epididymal tissue. Furthermore, the high level of mRNA lacking exon 9 in vas deferens cells reported by Teng *et al.*<sup>9</sup> suggested that those cells could be more sensitive to additional factors such as the V allele that may contribute to the decreased levels of normal transcripts. It has been shown that the V allele increases the proportion of transcripts lacking exon 9<sup>10</sup> and could thus be considered as a cis acting factor that contributes to decreasing the level of normal transcripts below the critical level which induces phenotypic expression.

Linkage disequilibrium alone is not a strong argument for a role of the M470V locus, since it could just indicate a third region close to this locus that might be implicated in exon 9 skipping regulation. However, no other polymorphism shows such linkage disequilibrium either in intron 8 or in exons 9 or 10. Thus, the M470V locus, so far considered an innocent polymorphism, should now be regarded as a possible modulating factor of CFTR expression.

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