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Mutation and linkage disequilibrium analysis in genetic counselling of Spanish cystic fibrosis families

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Abstract

We have analysed haplotypes for four DNA polymorphisms, closely linked to the cystic fibrosis (CF) gene, in 82 Spanish families, in which the CF probands are either homozygous for non-ΔF508 mutations or heterozygous for the ΔF508 deletion and other CF mutations. The analysis provides genetic data for a new polymorphism for the closely linked marker pKM.19, which is very strongly associated with CF. Haplotypes generated with the four marker loci are also in strong disequilibrium with the non-ΔF508 CF chromosomes. The data reported here are useful in 1 in 4 risk pregnancies of parents who have no living affected child, and when counselling close relatives of CF families who are negative for the major CF mutation. The data presented are useful in our population, in which the majority of CF mutations, apart from the ΔF508 deletion, are uncommon. For other populations in which mutation heterogeneity is also very high, it still might be more feasible to use RFLPs for diagnostic purposes, when analysis for common mutations is negative and DNA is available from the index patient. The experience presented here provides a model for these population groups who in turn should obtain their own haplotype data. In addition, the model system for genetic counselling presented here might also be useful for other genetic disorders.

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Genetic testing for cystic fibrosis (CF) has been considerably improved since the isolation of the CF gene and the discovery of the major CF mutation, which consists of a three nucleotide deletion (ΔF508) in the first putative ATP binding domain of the predicted protein (CF transmembrane conductance regulator, CFTR).[1–3]

The ΔF508 deletion has been found in approximately 70% of North American[3,4] and north European[5] CF chromosomes, but in only 50% of south Europeans.[6,7] Although several other mutations have been identified (CF Genetic Consortium), most of them are very uncommon,[8–10] and others have a frequency of approximately 3·5% in North Americans and north Europeans, but they seem to be even rarer in other populations.[8]

Haplotype analysis of CF chromosomes based on DNA markers closely linked to the disease locus suggests that the remainder of the CF mutant allele pool (non-ΔF508 mutations) in the Spanish population consists of various distinct mutations.[3,11] Mutation analysis provides, at the moment, full informativeness for prenatal diagnosis in only 25% (south European) to 55% (north European and North American) of couples.[4,12] Therefore, prenatal diagnosis and carrier detection of CF is currently performed by the analysis of the ΔF508 deletion or other defined mutations, and by using closely linked markers detecting RFLPs.[4,12] As the non-ΔF508 CF mutant allele pool is so heterogeneous, it may be more useful to use closely linked markers for genetic diagnosis of CF, when mutation analysis is negative for the common mutations.

We present here the haplotype analysis of ΔF508, non-ΔF508 CF chromosomes, and normal chromosomes, in CF families of Spanish origin, with four RFLPs closely linked to CF. A new RFLP analysed, pKM.19/*Scrf*I,[13] shows the highest non-random allelic association with CF (in both ΔF508 and non-ΔF508 CF chromosomes) described so far. The association between several haplotypes and non-ΔF508 chromosomes might be used to improve genetic analysis when DNA is not available from the CF proband and when counselling close relatives of CF families negative for common CF mutations.[14]

Materials and methods

DNA analysis was performed on a large series of families with members affected with CF. The families were referred to our centre in Barcelona between 1986 and 1989 from most of the regions of the country, and all were of Spanish origin. Diagnosis was confirmed by both the typical symptoms and two positive sweat tests. Only the families in which the CF patients were either homozygous for non-ΔF508 mutations or heterozygous for the ΔF508 deletion and other unknown CF mutations (in total 82 families) were considered for the present analysis.[11]

Genomic DNA from blood containing EDTA as anticoagulant was extracted from the parents, the CF patient, and in some cases from the grandparents. DNA was subjected to amplification as recommended by the manufacturer of *Taq* polymerase. Each 100 µl reaction mixture contained 50 mmol/l potassium chloride, 10 mmol/l Tris-hydrochloric acid (pH 7·8), 1·5 mmol/l magnesium chloride, 200 µmol/l of each deoxynucleotide triphosphate, 30 pmol/l of each oligonucleotide primer, 300 ng of genomic DNA, and 2·0 units of *Taq* polymerase; 50 µl of mineral oil was added to each reaction.

We studied four polymorphic loci: pXV-2c/*Taq*I, pKM.19/*Scr*fI, pKM.19/*Pst*I, and pMP6d-9/*Msp*I. The loci were analysed by restriction enzyme digestion after PCR amplification with the pairs of primers previously described[15–18] or by Southern blotting. Sequences for the amplification of the exon 10 region, containing the ΔF508 mutation, were from Riordan *et al.*[2] After an initial step of denaturing at 95°C for five minutes, 30 cycles were performed, including a 30 second denaturing step at 95°C, a 30 second annealing step as described, and one minute of polymerisation at 72°C. The last cycle was followed by a 10 minute step at 72°C. After amplification, 25 µl of sample was directly digested with the respective restriction enzyme. For the detection of the ΔF508 mutation, 5 µl of formamide–dye mixture (95% formamide/0·05% bromophenol blue/0·05% xylene cyanol/20 mmol/l EDTA) were added to 15 µl of the amplified DNA. Samples were loaded on to a 1 mm thick, 20 cm × 20 cm 6% PAGE (1·6 mol/l urea) in 1 × TBE buffer. Electrophoresis was performed at 400 V for two hours. Fragments of either 95 bp (ΔF508) or 98 bp were directly visualised using an UV transilluminator.

The degree of association between DNA markers and CF was measured by Yule's association coefficient. The standardised association $(A)=(ad-bc)/(ad+bc)$, where a, b, c, and d are the numbers of normal chromosomes with allele 1, CF non-ΔF508 with 1, normal with 2, and CF non-ΔF508 with 2, respectively.[3]

Results and discussion

Since the ΔF508 mutation accounts for only 50% of the Spanish CF chromosomes, and other mutations in the gene are very uncommon, the aim of this study was to evaluate the power of haplotype analysis when applied to genetic counselling and to provide more accurate carrier risk figures.

The results of haplotype analysis for pXV-2c, pKM.19, and pMP6d-9 in the 82 Spanish CF families in which the index patients are either homozygous for non-ΔF508 mutations or heterozygous for the ΔF508 deletion and another CF mutation are presented in table 1. The four loci analysed are situated at the 5′ end of the CF gene[1] in the following order: pXV-2c/*Taq*I, pKM.19/*Scr*fI, pKM.19/*Pst*I, and pMP6d-9/*Msp*I. Eleven haplotypes were found in total.

Haplotype data can be used to improve genetic counselling in the following situations: (1) couples

Table 1 Haplotype data for 82 Spanish CF families not homozygous or heterozygous for the ΔF508 mutation.

					Chromosomes					
					CF (non-ΔF508)		Normal		CF (ΔF508)	
Haplotype	T	S	P	M	No	%	No	%	No	%
a	1	1	2	2	39	34·2	7	4·3	47	94·0
b	2	1	2	2	2	1·8	1	0·6	1	2·0
c	2	1	1	1	0	0·0	1	0·6	0	0·0
d	1	2	1	2	0	0·0	5	3·0	1	2·0
e	1	2	2	2	11	9·7	13	7·9	0	0·0
f	1	2	1	1	21	18·4	47	28·7	0	0·0
g	1	1	1	1	0	0·0	1	0·6	0	0·0
h	2	2	2	2	7	6·1	26	15·9	1	2·0
i	2	2	1	1	30	26·3	58	35·4	0	0·0
j	2	2	1	2	4	3·5	3	1·8	0	0·0
k	2	2	2	1	0	0·0	2	1·2	0	0·0
Total					114		164		50	

T = pXV-2c/*Taq*I, S = pKM.19/*Scr*fI, P = pKM.19/*Pst*I, M = pMP6d-9/*Msp*I.

Table 2 Probability that a non-ΔF508 chromosome of a given haplotype is a CF chromosome [P(n-ΔF\a)].

Haplotype	Probability	Odds
a	0·0749	1 in 13·3
b	0·0282	1 in 35·5
c	0·0	—
d	0·0	—
e	0·0121	1 in 82·6
f	0·0064	1 in 156·2
g	0·0	—
h	0·00390	1 in 256·4
i	0·00747	1 in 133·9
j	0·0190	1 in 52·6
k	0·0	—

with less than a 1 in 4 risk, and (2) couples with a 1 in 4 risk and a dead CF child, who is negative for the ΔF508 mutation.

HAPLOTYPE ANALYSIS IN COUPLES WITH LESS THAN 1 IN 4 RISK

The probability that a chromosome of a given haplotype (for example, haplotype **a**) is a non-ΔF508 CF chromosome [P(n-ΔF\a)] can be calculated from the data in table 1. This probability is based on a Bayesian calculation, and takes into account the fact that the haplotype is found in a phenotypically normal subject:

$$P(\text{n-}\Delta F\backslash \mathbf{a}) = \frac{F_a \times F_{\text{n-}\Delta F}}{F_a \times F_{\text{n-}\Delta F} + F'_a}$$

where F_a is the frequency of haplotype **a** in the non-ΔF508 CF chromosomes, $F_{\text{n-}\Delta F}$ is the gene frequency of the non-ΔF508 mutations in the population (1/100 in the Spanish population), and F'_a is the frequency of haplotype **a** in the normal chromosomes after testing for the ΔF508 mutation.

Table 2 shows the probabilities calculated for all the haplotypes found in the Spanish population. Four haplotypes (**c**, **d**, **g**, and **k**) were not found in this sample of 114 non-ΔF508 CF chromosomes; however, these haplotypes represent only 6% of the overall normal chromosomes. The probabilities that a non-ΔF508 chromosome with a given haplotype is a CF chromosome are considerably reduced in the case of haplotypes **f**, **h**, and **i**, with odds of 1 in 156·2, 1 in 256·4, and 1 in 133·9, respectively. For haplotypes **a**, **b**, **e**, and **j**, the probabilities of being CF chromosomes are increased to 1 in 13·3, 1 in 35·5, 1 in 82·6, and 1 in 52·6, respectively. Thus, the probability of a haplotype derived from a parent with no family history of CF and not carrying the ΔF508 mutation can be ascertained using these data.

The probability that a phenotypically normal subject with genotype **ab** is a carrier of a non-ΔF508 mutation can be calculated as follows:

$$P(\text{C n-}\Delta F\backslash \mathbf{ab}) = \frac{(F_b \times F'_a + F'_b \times F_a) \times F_{\text{n-}\Delta F}}{(F_b \times F'_a + F'_b \times F_a) \times F_{\text{n-}\Delta F} + (F'_b \times F'_a)}$$

where F_b and F'_b are the frequencies of haplotype **b** in their respective non-ΔF508 and normal chromosome populations.

Table 3 shows the probabilities of carrying a non-ΔF508 mutation for all 66 expected genotypes of phenotypically normal subjects in the Spanish population. Considering a carrier frequency for a non-ΔF508 mutation of 1 in 50, this figure is notably improved for some genotypes (for example, for genotypes **df** 1 in 155 and **dh** 1 in 256·4). For other genotypes the probabilities of being a carrier are considerably increased (**aa** 1 in 7·2, **ab** 1 in 10·1, and **ad** 1 in 13·3). Other genotypes only show slight modifications of the previous carrier risk figures.

Table 3 Probability that a person of a particular genotype is a carrier of a non-ΔF508 mutation [P(C n-ΔF\genotype)].

Genotype	Risk	Genotype	Risk	Genotype	Risk
aa	1 in 7·2	**cd**	—	**ek**	1 in 82·6
ab	1 in 10·1	**ce**	1 in 82·6	**ff**	1 in 78·1
ac	1 in 13·3	**cf**	1 in 155·0	**fg**	1 in 155·0
ad	1 in 13·3	**cg**	—	**fh**	1 in 97·1
ae	1 in 11·7	**ch**	1 in 256·4	**fi**	1 in 72·5
af	1 in 12·4	**ci**	1 in 134·0	**fj**	1 in 39·7
ag	1 in 13·3	**cj**	1 in 52·6	**fk**	1 in 155·0
ah	1 in 12·8	**ck**	—	**gg**	—
ai	1 in 12·3	**dd**	—	**gh**	1 in 256·4
aj	1 in 11·0	**de**	1 in 82·6	**gi**	1 in 134·0
ak	1 in 13·3	**df**	1 in 155·0	**gj**	1 in 52·6
bb	1 in 18·2	**dg**	—	**gk**	—
bc	1 in 35·5	**dh**	1 in 256·4	**hh**	1 in 128·9
bd	1 in 35·5	**di**	1 in 134·0	**hi**	1 in 88·5
be	1 in 70·4	**dj**	1 in 52·6	**hj**	1 in 44·0
bf	1 in 29·1	**dk**	—	**hk**	1 in 256·4
bg	1 in 35·5	**ee**	1 in 41·7	**ii**	1 in 67·6
bh	1 in 31·3	**ef**	1 in 54·3	**ij**	1 in 38·2
bi	1 in 28·3	**eg**	1 in 82·6	**ik**	1 in 134
bj	1 in 21·6	**eh**	1 in 62·9	**jj**	1 in 26·8
bk	1 in 35·5	**ei**	1 in 51·5	**jk**	1 in 52·6
cc	—	**ej**	1 in 32·6	**kk**	—

The typical situation in which haplotype information could improve the carrier risk figure is for couples with less than a 1 in 4 risk of CF (for example, couples consisting of a known carrier and a person from the general population), where the low risk parent does not have the ΔF508 mutation. The risk of the partner being a carrier is modified from 1 in 25 to 1 in 50 after mutation analysis. Haplotype analysis including the pKM.19/*Scrf*I marker allows the modification of their risk of being a carrier for a non-ΔF508 mutation. A final carrier risk could be given, which ranges from 1 in 7·2 to 1 in 256·4, and for some genotypes it is practically zero. Thus, for the couple, the risk of having a CF child, calculated by mutation analysis is 1 in 200, whereas using haplotype analysis the risk can be further modified to between 1 in 28·8 and 1 in 825·6, and in some cases to practically zero.

HAPLOTYPE ANALYSIS IN COUPLES WITH A 1 IN 4 RISK OF CF

When counselling parents of a dead child for a further pregnancy, the probabilities for CF of each possible genotype in the fetus are calculated from haplotype data in the parents using Bayes's theorem. The calculations are performed for each possible phase in each parent, under the assumption that both parents are obligate carriers of a CF mutation. Considering one parent with genotype **ab**, the probabilities that the CF mutation is associated either with haplotype **a** or with haplotype **b**, given the parent is **ab**, are:

$$P(CF_a \backslash \mathbf{ab}) = \frac{F_a \times F'_b}{F_a \times F'_b + F'_a \times F_b}$$

or

$$P(CF_b \backslash \mathbf{ab}) = 1 - P(CF_a \backslash \mathbf{ab}).$$

Considering that the other parent has a genotype **cd**, then the probabilities that the CF mutation is associated either with **c** or **d** are: $P(CF_c \backslash \mathbf{cd})$ or $P(CF_d \backslash \mathbf{cd}) = 1 - P(CF_c \backslash \mathbf{cd})$.

The probabilities for each possible genotype are the products of the probabilities that each haplotype contributed by each parent carries a CF mutation:

$P(CF_{ac} \backslash \mathbf{ab}$ and $\mathbf{cd}$ in parents$) = P(CF_a \backslash \mathbf{ab}) \times P(CF_c \backslash \mathbf{cd})$
$P(CF_{bc} \backslash \mathbf{ab}$ and $\mathbf{cd}$ in parents$) = P(CF_b \backslash \mathbf{ab}) \times P(CF_c \backslash \mathbf{cd})$
$P(CF_{ad} \backslash \mathbf{ab}$ and $\mathbf{cd}$ in parents$) = P(CF_a \backslash \mathbf{ab}) \times P(CF_d \backslash \mathbf{cd})$
$P(CF_{bd} \backslash \mathbf{ab}$ and $\mathbf{cd}$ in parents$) = P(CF_b \backslash \mathbf{ab}) \times P(CF_d \backslash \mathbf{cd})$.

These calculations can also be used in the case where one of the parents carries a known mutation (for example, ΔF508 associated with haplotype **a**) and that this mutation is present in the fetal genotype. In this case, the probability of the fetus being CF is equal to the probability that the haplotype contributed by the parent carries a non-ΔF508 mutation:

$P(CF_{ac} \backslash \mathbf{ab}$ and $\mathbf{cd}$ in parents$)$ (when **a** is CF$) = P(CF_c \backslash \mathbf{cd})$.

Finally, the risk for a further pregnancy could also be modified if the couple has a phenotypically normal child.

Although when counselling for a further pregnancy in obligate CF carriers the risk figures obtained for some genotypes are as good, or even better, than those obtained with microvillar enzyme (MVE) analysis alone, for most cases haplotype analysis should be combined with MVE in a Bayesian calculation. Haplotype data are entered as the 'prior probability' in the couple for a fetus with CF and MVE data as the 'conditional probability'.[14]

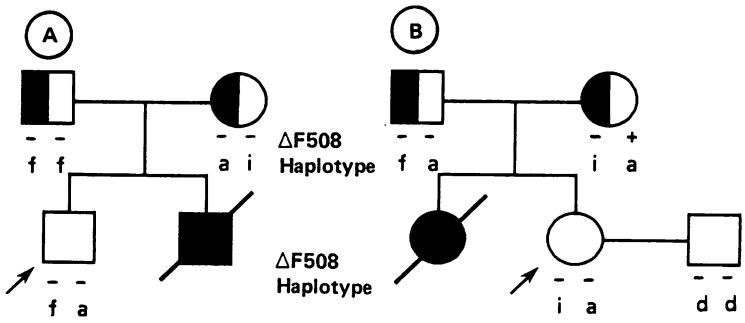
In the case of the sibs of a dead CF child, for whom no genetic material is available, and whose parents are negative for the ΔF508 mutation, their risk of carrying a CF mutation could be calculated using the linkage disequilibrium data in table 1 and Bayes's theorem.[14 19 20] The probability that a child with a normal phenotype and whose parents are obligate carriers is a carrier can be calculated as follows:

$$P(C_{ad} \backslash \mathbf{ab} \text{ and } \mathbf{cd} \text{ in parents}) = \frac{P(CF_{ac}) + P(CF_{bd})}{P(CF_{ac}) + P(CF_{bc}) + P(CF_{bd})}.$$

In the case that only one of the chromosomes (for example, haplotype **a**) in the phenotypically normal subject is at risk of carrying a CF mutation (that is, when a person is known to have inherited at least one normal chromosome from his/her parents), the probability that that person is a carrier is:

$P(C_{ad} \backslash \mathbf{ab}$ and $\mathbf{cd}$ in parents$)$ (when **d** is normal$) = P(CF_a \backslash \mathbf{ab})$.

Two examples of the application of haplotype analysis in genetic counselling are presented in the figure. The index patient in pedigree A is the brother of a dead CF child and we want to calculate the risk of his carrying a CF mutation. The family is negative for the ΔF508 mutation. Haplotypes are ascertained for the family, and the carrier risk of the subject is estimated according to his genotype (**fa**):



ΔF508 mutation and haplotype analysis in two CF families (A and B) with a dead CF patient. The index patient is indicated by an arrow. − = non-ΔF508 mutation, + = ΔF508 mutation. Haplotypes are from table 1.

$$P(C_{fa} \backslash \mathbf{ff} \text{ and } \mathbf{ai})$$

$$= \frac{P(CF_{fi}) + P(CF_{fa})}{2 \times P(CF_{fi}) + P(CF_{fa})}$$

$$= 0{\cdot}50/0{\cdot}54 = 0{\cdot}92$$

and is modified from 2 in 3 to 1 in 1·1. Another example is shown in pedigree B. The sister of a dead CF patient has married into the general population. The couple are negative for the ΔF508 mutation, so she has not inherited ΔF508 from her mother, but she may still carry the CF mutation inherited from her father. The haplotype (**a**) that she has inherited from her father has a high risk (92·5%) of being a CF chromosome. On the other hand, the typing for her husband shows that he is homozygous for a haplotype (**d**) not present in CF chromosomes. Thus, although she has a high risk of carrying a CF mutation (1 in 1·1), the risk of the couple for CF is negligible. However, if her husband had an **aa** genotype, then the risk that the couple had a CF child would be:

$$1/4 \times P(\text{C n-}\Delta\text{F} \backslash \mathbf{aa}) \times P(CF_{a} \backslash \mathbf{af}) =$$

$$1/4 \times 1/7{\cdot}2 \times 1/1{\cdot}1 = 1/31.$$

LINKAGE DISEQUILIBRIUM

The degree of association between the DNA markers studied here and CF, as measured by Yule's association coefficient, is shown in table 4. Strong allelic association was detected with all RFLPs, but the highest degree of association with CF was found with pKM.19/*Scrf*I, an allelic system situated 125 kb from the CF gene. The lower disequilibrium detected with markers which are closer to the CF gene (pKM.19/*Pst*I and pMP6d-9/*Msp*I) is probably the result of the variation in the allelic distribution among the normal chromosomes. pKM.19/*Scrf*I also shows higher association with CF than markers not analysed here, but tested by others.[3] This is true for several intragenic markers that are located near the major CF mutation,[3] reflecting the influence of allelic distribution among normal chromosomes in the values of disequilibrium obtained. Data for the KM.19/*Scrf*I allelic system in other populations (British, Italian, and German) also show a high degree of association with CF (Ramsay, Novelli, and Stutman, personal communications). Particularly interesting is the high disequilibrium coefficient value (A = 0·80) obtained with pKM.19/*Scrf*I in non-ΔF508 chromosomes, suggesting that other common mutations should be present in the Spanish population.

Table 4 Values of linkage disequilibrium for RFLPs associated with the CF locus for non-ΔF508 and ΔF508 CF chromosomes.

Allelic system	Association (A)	
	non-ΔF508	ΔF508
pXV-2c/*Taq*I	0·35	0·93
pKM.19/*Scrf*I	0·80	0·99
pKM.19/*Pst*I	0·43	0·98
pMP6d-9/*Msp*I	0·42	1·00

A = Yule's association coefficient values obtained from allelic distribution shown in table 1 (see text).

The haplotype in which ΔF508 arose (**a**) is also the commonest haplotype (34·2%) in the non-ΔF508 CF chromosomes; this haplotype is present in only 4·3% of normal chromosomes. Thus, several mutations have arisen in the same rare haplotype. The preferential association between this uncommon haplotype and CF mutations is not well understood. We do not know what the haplotype distribution was in normal chromosomes several thousand years ago in the population in which these CF mutations occurred. It might be that haplotype **a** was quite common in the population in which the major CF mutations originated, but hypotheses regarding selective enhancement for mutations in this haplotype should be contemplated.

Haplotype data, in order to obtain carrier risk modifications in the cases with no DNA available from the CF index patient, should be obtained for each national population. The use of particular

haplotype data for the different ethnic groups is still more crucial, as in the case of the Basque population in Spain, where approximately 85% of CF chromosomes carry the ΔF508 mutation.[21]

Although several intragenic polymorphisms have been identified, they are not very informative, mainly because they show strong allelic association with other markers at the CF locus.[3] The markers at the *D7S23* locus (XV-2c and KM.19) are still playing a relevant role in genetic diagnosis of CF.[22-24] A large body of data has been generated during the last three years for these markers, and recombinational events have been documented,[25] although some have been withdrawn,[26] with almost no recombination at the KM.19 locus. The new marker described here increases the power of linkage disequilibrium in haplotype analysis when testing cases in which one or both CF mutations have not been identified. The association between alleles at the pKM.19/*Scrf*I locus and CF markedly improves genetic analysis in these situations.

The data presented here are useful in our population, in which the majority of CF mutations, apart from the ΔF508 deletion, are uncommon. Data on the frequency of the major CF mutation in several south European populations, and the large mutation heterogeneity found in the non-ΔF508 mutant allele pool, suggest that RFLP and haplotype analysis may be the method of choice for genetic analysis in several circumstances. This is particularly true for all the Mediterranean countries, where it is estimated that only one-third of the 75 000 CF families from this region are expected to be fully informative for mutation analysis.[12] Therefore, for each population, data should be obtained and analysed in order to provide useful risk modifications in the different situations. However, it is expected that mutations covering at least 5% of CF chromosomes each will be detected in some populations and that mutation tests could be developed that cover a large proportion of CF cases. If this is the case, linkage disequilibrium data will not be used in the way that has been shown here. However, if the number of mutations is very high, it still might be more feasible to use RFLPs for diagnostic purposes when mutation analysis for common mutations is negative and DNA is available from the index patient.

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