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Association between Haplotypes and Specific Mutations in Swiss Cystic Fibrosis Families

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ABSTRACT. Cystic fibrosis (CF) is the most common severe autosomal recessive genetic disorder in Caucasian populations, with an incidence of about 1 in 2000 live births, implying a carrier frequency of about 1 in 22. In 1989, the CF gene was isolated and characterized and the major mutation (ΔF508), a 3-bp deletion that results in the loss of a phenylalanine residue at position 508, was detected. To determine the frequency of the ΔF508 mutation and the predicted number of additional mutations in our population, we have undertaken a collaborative study of 215 CF patients and 175 CF parents in Switzerland. The ΔF508 mutation in exon 10 has been found in 70% of the CF chromosomes, and the exon-11 mutation R553X seems to be the second most common CF mutation in our population, with a frequency of 5.3%, whereas the G551D mutation (also in exon 11) has not been detected at all. Haplotype determination of 430 CF and 175 normal chromosomes using XV-2c, KM19, MP6d-9, and J3.11 has been proven to be very helpful in providing additional carrier risk calculations: Haplotypes 1 (1221), 2 (1222), 6 (2111), and 7 (2221) increase the risk of being a carrier from 1 in 55 (haplotype 6) to 1 in 17 (haplotype 1), whereas haplotypes 3 (1122), 4 (1112), 8 (2222) and 10 (1111) lower the risk from 1 in 144 (haplotype 3) to 1 in 1678 (haplotype 10). Moreover, the mutation R553X shows strong correlation with haplotype 3, leading to the suggestion that haplotypes 1, 2, 5, and 6 may account for four additional mutations in Switzerland. It is concluded that haplotype analyses should be offered to partners of individuals with a family history of CF, giving a more informative carrier risk estimation than the global 1 in 80. For couples at increased risks, where no completely reliable prenatal tests exist, microvillar enzyme testing in combination with DNA analysis is recommended. (*Pediatr Res* 30: 304–308, 1991)

Abbreviations

CF, cystic fibrosis
RFLP, restriction fragment length polymorphism
PCR, polymerase chain reaction

CF is the most common severe autosomal recessive genetic disorder in Caucasian populations, with an incidence of about 1 in 2000 live births, implying a carrier frequency of about 1 in 22. Its severity of expression in patients varies widely, and the disease involves multiple organ systems. Many of the symptoms of cystic fibrosis are associated with defective transport of chloride ions across epithelia, suggesting that the CF gene product, called the CF transmembrane conductance regulator, might be involved in the regulation or structure of the chloride channel (1). A review of the physiologic and molecular aspects of CF has recently been published (2).

One of the major breakthroughs in CF research was the mapping of the gene to chromosome 7q21-7q31 by genetic linkage analysis (3–5). Subsequently, DNA sequences progressively closer to the gene were identified (6–8). DNA polymorphisms presenting allelic association with the CF gene (linkage disequilibrium) were found, implying that the majority of the CF chromosomes arose from one or a few mutational events (9–11). RFLP analysis of families that showed recombination between the markers and the disease, plus long-range restriction mapping, located the gene to a region spanning approximately 500 kb (12–15). In 1989, the gene was finally cloned. It is predicted to encode a protein of 1480 amino acids; the major mutation ΔF508, a 3-bp deletion that results in the loss of a phenylalanine residue at position 508, has been defined (16–18). The frequency of ΔF508 has found to be 68–78% in North American (18), British, and Dutch patients (19), whereas in Spanish and Italian populations and in black American patients the deletion is present in only 51–58% (19) and 37% (11) of the CF chromosomes, respectively. So, it seems likely there will be a large number of CF mutations including deletions (17), frameshift mutations (20), and point mutations (21, 22). More than 100 mutations have been detected so far, most of which are rarely found. However, two rather common CF mutations localized in exon 11 have recently been described by Cutting *et al.* (22): The G551D mutation, causing an amino-acid substitution (G1784→A; Gly551→Asp), and the nonsense mutation R553X, creating a premature termination signal (C1789→T; Arg553→Stop). The ability to detect CF mutations at the DNA level offers the opportunity of more precise carrier detection and prenatal diagnosis. But effective DNA-based testing requires the definition of all CF mutations and their frequencies for individual populations because of frequency variations among different geographic locations.

We previously reported on DNA marker haplotypes of RFLP flanking the CF gene in CF families of Switzerland (23). The aim of this study was, one, to improve in our country carrier testing, prenatal diagnosis, and risk calculations by combining direct mutation analyses and haplotype information. Two, we wanted

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to contribute further information on mutation and haplotype frequencies to the CF mutation research in Europe.

To determine the ΔF508-deletion frequency and the number of additional mutations in our population, we have undertaken a collaborative study of the CF patients in Switzerland. Here, we report the results of ΔF508-, R553X-, and G551D-screening by PCR analysis and the haplotype distribution of CF and normal chromosomes that has been generated by RFLP analyses using the markers XV-2c, KM19, MP6d-9, and pJ3.11.

MATERIALS AND METHODS

Subjects. We studied 430 CF chromosomes, from 215 patients in whom the diagnosis of CF met standard criteria (24), obtained from the five university genetic centers in Switzerland. In addition, 175 normal chromosomes from 175 parents heterozygous for CF have been analyzed for haplotype determination. All patients and parents are native Swiss from central, western, northern, and eastern Switzerland, mostly of Celtic and Germanic origin.

DNA analyses. DNA extraction from whole blood containing EDTA as an anticoagulant, restriction enzyme digestion, Southern blotting, and hybridization using oligo-labeled probes were performed as described before (23).

RFLP were determined for the XV-2c/*Taq*I, (8), KM19/*Pst*I (25), MP6d-9/*Msp*I (26), and J3.11/*Msp*I (4) probes and haplotypes were constructed using the CF patients' genotypings to establish phase.

The ΔF508 deletion was detected by amplification of genomic DNA by PCR (27) followed by vertical electrophoresis in a polyacrylamide gel and ethidium bromide staining (28). The PCR primer sequences were 5′-GTTTTCCTGGATTATGCCTGGCAC-3′ and 5′-GTTGGCATGCTTTGATGACGCTTC-3′ (17).

The amplification product of the normal allele is 98 bp and of the deleted allele is 95 bp. Two hundred pmol of each primer were used with 200–300 ng of genomic DNA and 5.0 U *Taq*I-polymerase (Perkin-Elmer Cetus, Kuesnacht, Switzerland) in a total volume of 100 μL under paraffin oil. Annealing conditions were 64°C for 90 s, extension at 72°C for 120 s, and denaturation at 94°C for 60 s, for 28 cycles with a final cycle of 5 min for extension in a Perkin-Elmer Amplification System. About one fifth of the amplification product was applied on a 20-cm 12% polyacrylamide gel and run at 90 V in Tris-borate/EDTA buffer over night. Fragments were visualized and photographed after staining with ethidium bromide and illuminating them with 306 nm UV light.

The R553X mutation was detected by PCR amplification using exon-11 primers 11i-5′-CAACTGTGGTTAAAGCAATAGTGT and 11i-3′-GCACAGATTCTGAGTAACCATAAT (22), resulting in a 425-bp fragment. *Hin*cII digestion of genomic DNA amplified from normal exon-11 sequence produces fragments of 186 and 239 bp. The R553X as well as the G551D mutation cause the loss of the normal *Hin*cII recognition site. Normal exon-11 amplification products cannot be cut with *Mbo*I, whereas the G551D mutation creates a new *Mbo*I-site, resulting in two fragments of 182 and 243 bp.

DNA of non-ΔF508-CF chromosomes was amplified with exon-11 primers and subsequently cut with *Hin*cII. Amplification products that showed no *Hin*cII restriction site were additionally cut with *Mbo*I. PCR and gels were performed as described for ΔF508 analyses.

The mutations were confirmed by dideoxy sequencing (29) on single strand templates generated by asymmetric reamplification, using an internal primer located 3′ to exon 11 (Malik NJ, Hofmann S, Reiser P, Bühler EM, unpublished experiment). Asymmetric amplification was carried out using 2 μL of the initial amplification products, an amplification mixture containing 1 pmol of primer 11i-5, and 50 pmol of primer 11int-3 (5′-GTGATTCTTAACCCACTAGCC-3′). The resultant single strand DNA was purified from primers and enzyme by alcohol precipitation and one fifth of the total used for sequencing with the T7 DNA sequencing kit (Pharmacia, Dübendorf, Switzerland). Twenty pmol of the primer 11i-5 was used as the sequencing primer.

Sequence details have generously been provided by members of the international CF genetic analysis consortium.

Risk calculations. Carrier risks for non-ΔF508 chromosomes of particular haplotypes were calculated by combined statistical analysis including the Hardy-Weinberg equilibrium and the Bayes' theorem according to Lemna *et al.* (30) and Watson *et al.* (31) using the formula $R(x) = (pc/C):[pc/C + (1 - p)n/N]$, where p = prior risk that a chromosome carries the CF gene (1/40), c = number of non-ΔF508, non-R553X CF chromosomes carrying haplotype x, C = total number of CF chromosomes (430), n = number of normal chromosomes with haplotype x, and N = number of normal chromosomes (175).

RESULTS

ΔF508-deletion. We analyzed 430 CF chromosomes for the presence of ΔF508 and found the frequency of the deletion to be 70% (301 out of 430). One hundred nine (51%) of the 215 patients were found to be homozygous for the ΔF508 mutation, 83 (38%) were compound heterozygous, and 23 (11%) did not bear the deletion on either chromosome, being either compound heterozygotes for two unknown mutations or homozygotes for one not yet defined mutation.

R553X and G551D mutations. The remaining 30% of CF chromosomes (129 out of 430) without the ΔF508 mutation have been amplified with the primer pair of exon 11 followed by *Hin*cII digestion. DNA that was not cut, demonstrating loss of the restriction site, was additionally digested by *Mbo*I to test it for G551D. After confirmation by sequencing, all of our exon 11 mutations turned out to be R553X mutations. The R553X mutation has been detected in 23 (5.35%) of the 430 CF-chromosomes and in 23 (18%) of the 129 non-ΔF508 chromosomes. Three patients showed homozygosity for R553X, whereas 17 were compound heterozygotes. In 14 of these 17 patients, the R553X mutation was paired with the ΔF508 deletion.

Haplotypes. The marker allele constellations for XV-2c/KM19/MP6d-9/J3.11 have been determined in 430 CF chromosomes and in 175 normal chromosomes and were expected to occur in 16 different haplotypes. The presence or absence of the ΔF508 deletion was correlated with haplotypes, and the haplotype distributions for non-ΔF508 CF chromosomes as well as for normal chromosomes are presented in Figures 1 and 2 (only 11 out of 16 haplotypes have been found).

Figure 1 shows the haplotype distributions of 310 ΔF508-bearing CF chromosomes and 129 non-ΔF508 CF chromosomes: 70.4% (212 out of 301) of the ΔF508-bearing chromosomes are associated with haplotype 1 (1221); 27.3% (82 out of 301) are associated with haplotype 2 (1222); and four and three chromosomes, respectively, show haplotypes 11 (1121) (1.3%) and 8 (2222) (1%). The data confirm that the ΔF508 deletion is strongly associated with the marker allele constellation 1221, which has previously been reported to be the most common haplotype among CF chromosomes in Caucasians (9–11, 20). However, the fact that the ΔF508 deletion was not found on all CF chromosomes carrying haplotype 1 and the observation of unequal distribution of the other haplotypes suggested additional moderately common mutations correlated with particular haplotypes in our patients. The haplotype distribution of the non-ΔF508 CF chromosomes is more heterogeneous: The most common haplotype is number 3 (1122), detected in 25 (19.4%) of the 129 CF chromosomes without the ΔF508 mutation and strongly linked to the exon-11 mutation R553X. Every R553X-bearing chromosome had the haplotype 3, and, conversely, all but two haplotype 3 chromosomes showed the R553X mutation. Haplotypes 1 (21 out of 129) and 2 (17 out of 129) as well as 5

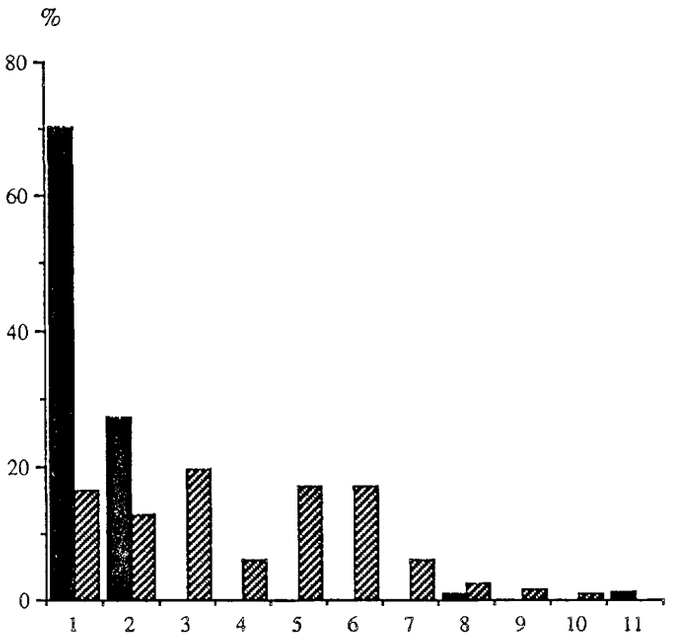

Fig. 1. Haplotype distributions of 301 ΔF508 chromosomes (■) and 129 non-ΔF508 CF chromosomes (▨). *1–11*, haplotypes generated from allele constellations of the markers XV-2c/KM19/MP6d-9/J3.11. *1*, 1221; *2*, 1222; *3*, 1122 (correlated with R553X); *4*, 1112; *5*, 2112; *6*, 2111; *7*, 2221; *8*, 2222; *9*, 2122; *10*, 1111; *11*, 1121.

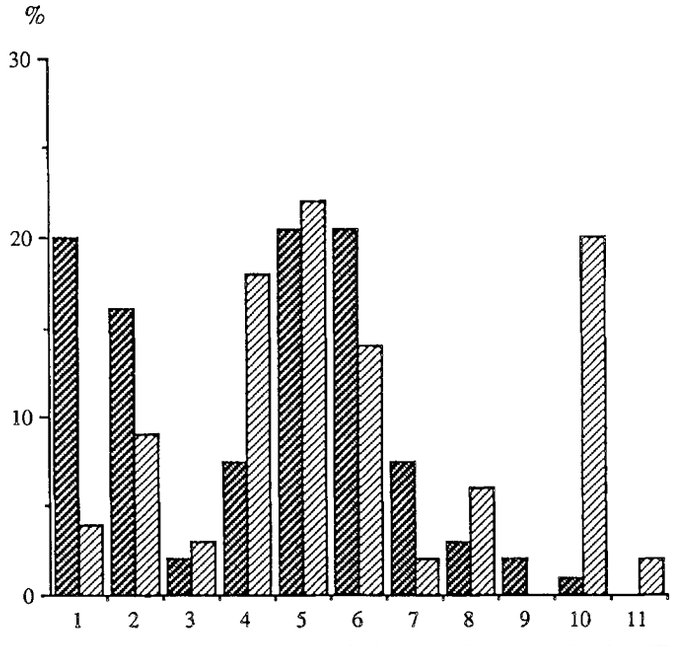

Fig. 2. Haplotype distributions of 106 non-ΔF508, non-R553X CF chromosomes (▨) and 175 normal chromosomes (▨). *1–11*, haplotypes generated from allele constellations of the markers XV-2c/KM19/MP6d-9/J3.11. *1*, 1221; *2*, 1222; *3*, 1122; *4*, 1112; *5*, 2112; *6*, 2111; *7*, 2221; *8*, 2222; *9*, 2122; *10*, 1111; *11*, 1121.

(22 out of 129) and 6 (22 out of 129) are also rather frequent, occurring in 13–17% of non-ΔF508 chromosomes; they may be specifically correlated with four additional mutations. Haplotypes 4 and 7 are present in eight of 128 non-ΔF508 CF chromosomes each, whereas haplotypes 8 (three out of 129), 9 (two out of 129), and 10 (one out of 129) are rarely found.

Figure 2 demonstrates the haplotype distributions of normal chromosomes *versus* CF chromosomes without the ΔF508 deletion and without the R553X mutation. Normal chromosomes seem to be preferably associated with haplotypes 5 (38 out of 175; 22%), 10 (35 out of 175; 20%), 4 (31 out of 175; 18%), and 6 (25 out of 175; 14%), whereas haplotypes 2 (15 out of 175; 9%), 8 (10 out of 175; 6%), 1 (seven out of 175; 4%), and 3 (six out of 175; 3%) are represented in lower frequencies and haplotypes 7 and 11 are rarely found (four out of 175; 2%). Moreover, haplotypes 12–16 (1211/1212/2121/2212/2211) have not been detected at all, either in CF or in normal chromosomes. When comparing the haplotype frequencies of normal and non-ΔF508, non-R553X chromosomes, haplotypes 10, 4, 3, and 8 indicate a normal chromosome with a very high probability. In contrast, the risk of carrying a CF mutation is obviously increased in the presence of haplotypes 1, 2, 6, and 7.

Table 1 shows the risks for non-ΔF508, non-R553X chromosomes of specific haplotypes to be CF chromosomes and the carrier risk calculations for individuals of specific non-ΔF508, non-R553X genotypes.

DISCUSSION

Previous results provided by the original North American study (18) and data reported by other groups (19) indicated that the ΔF508 deletion is present in more than two thirds of CF chromosomes in Northern Europe. We have analyzed 215 patients from Switzerland and found the ΔF508 frequency to be 70%. This finding supports the hypothesis that the predominant CF mutation, present throughout Europe, originated in a specific location before spreading according to a northwest-southeast gradient to all populations by migration (19, 32).

Examination of exon 11 revealed only one of the four mutations described by Cutting *et al.* (22), R553X. This nonsense mutation was found on 23 of 129 non-ΔF508 CF chromosomes, resulting in a frequency of 18%. It therefore seems to be the second most common CF mutation in our population. Cutting *et al.* (22) reported the G551D mutation (which we did not detect at all) to be found in 4% of CF chromosomes of Caucasian and the R553X mutation to be found in 5% of CF chromosomes of black Americans. Provisional data from different European countries show 0.5–13% of the non-ΔF508 CF chromosomes carrying the G551D mutation and 1–10% of the non-ΔF508 CF

Table 1. *Risks for non-ΔF508, non-R553X chromosomes to be CF-chromosomes and carrier risks for individuals of specific non-ΔF508, non-R553X genotypes*

Haplo-type	Risk	Geno-type	Carrier risk	Geno-type	Carrier risk
		1/1	1 in 17	3/10	1 in 265
1	1 in 33	1/2	1 in 24	4/4	1 in 186
2	1 in 86	1/3	1 in 29	4/5	1 in 115
3	1 in 288	1/4	1 in 30	4/6	1 in 85
4	1 in 372	1/5	1 in 27	4/7	1 in 43
5	1 in 167	1/6	1 in 25	4/8	1 in 172
6	1 in 110	1/7	1 in 20	4/10	1 in 335
7	1 in 49	1/8	1 in 30	5/5	1 in 83
8	1 in 320	1/10	1 in 33	5/6	1 in 66
10	1 in 3357	2/2	1 in 43	5/7	1 in 38
		2/3	1 in 66	5/8	1 in 110
		2/4	1 in 70	5/10	1 in 159
		2/5	1 in 57	6/6	1 in 55
		2/6	1 in 48	6/7	1 in 34
		2/7	1 in 31	6/8	1 in 82
		2/8	1 in 68	6/10	1 in 106
		2/10	1 in 84	7/7	1 in 25
		3/3	1 in 144	7/8	1 in 42
		3/4	1 in 162	7/10	1 in 48
		3/5	1 in 106	8/8	1 in 160
		3/6	1 in 80	8/10	1 in 292
		3/7	1 in 42	10/10	1 in 1678
		3/8	1 in 152		

chromosomes carrying the R553X mutation (personal communication from the European Community Cystic Fibrosis Consortium).

Identification of the ΔF508 and R553X mutations allow the detection of 75% of CF chromosomes in Switzerland, raising the question of population-based screening for carriers. The detection of multiple individually rare mutations (more than 100 at the time of writing) makes carrier testing more and more difficult, and, if no more than 95% of carriers can be detected, screening may not be advisable (33, 34). However, carrier testing that is nearly 100% informative can be offered to all individuals with a family history of CF, inasmuch as carrier detection can be performed by linkage analyses in addition to mutation analyses.

The association between DNA polymorphism haplotypes and specific gene mutations has been proven to be of great value in a variety of autosomal recessive disorders. Therefore, we determined the haplotypes of 301 ΔF508 and 129 non-ΔF508 CF chromosomes and 175 normal chromosomes using three DNA markers (XV-2c, KM19, MP6d-9) centromeric and one marker (J3.11) telomeric to the CF gene. The ΔF508 deletion was almost exclusively associated with haplotypes 1 (70.5%) and 2 (27%), which have previously been found to occur most commonly in CF chromosomes (10, 11, 23). Haplotype frequencies of the non-ΔF508 CF chromosomes may differ from country to country depending on ethnic background, for example racial admixture, and on the location and the moment when a new mutation occurred. The strong correlation of the R533X mutation with haplotype 3 suggests that haplotypes 1, 2, 5, and 6 may be associated with at least four other specific mutations. As long as these mutations are not known, haplotype frequencies can be used for carrier risk calculations.

Screening for ΔF508 and R553X detects the mutation in 75% of CF chromosomes in Switzerland. If a non-related partner of an individual heterozygous for CF tests negative for ΔF508 and R553X, his risk of being a carrier decreases from the population risk of 1 in 20 down to 1 in 80. Combination with haplotype data provides still more accurate calculations: Table 1 shows the risk that a non-ΔF508, non-R553X chromosome of a particular haplotype is a CF chromosome and the carrier risks for individuals of specific non-ΔF508, non-R553X genotypes. The data demonstrate that haplotypes generated from the marker allele constellation of XV-2c, KM19, MP6d-9, and J3.11 influence strongly carrier risk calculations. Haplotypes 1, 2, 6, and 7 increase the risk of being a carrier from 1 in 55 (haplotype 6) to 1 in 17 (haplotype 1), whereas haplotypes 3, 4, 8, and 10 lower the risk from 1 in 144 (haplotype 3) to 1 in 1678 (haplotype 10). However, the global risk of 1 in 80 is not markedly changed by haplotype 5, inasmuch as it shows approximately the same frequency in normal and non-ΔF508 chromosomes. Mutations on chromosomes carrying haplotype 5 may be of more recent origin. Thus, haplotype analyses that give a more informative risk estimation than the global 1 in 80 should be offered to partners of individuals with a family history of CF. Where one partner is a known carrier and the other has genotype 4/4, the risk in any pregnancy decreases from 1 in 320 to 1 in 744, whereas for genotype 1/1 the risk increases to 1 in 68. For couples at increased risk, there are no completely reliable prenatal tests; however, microvillar enzyme testing in combination with DNA analysis is recommended in this situation and will provide the most help possible to the couple in deciding whether they want the pregnancy to be interrupted or continued.

On the basis of PCR analyses for ΔF508 and R553X in conjunction with DNA marker haplotype comparisons, we conclude that there are at least four additional CF mutations in our population. Three of our CF patients have been found to be homozygous for R553X; they present with normal weight and low Crispin Norman scores up to the age of 10 and rapid progression from the age of 12, suggesting a two-stage course. Further studies on clinical symptoms in correlation with haplotypes are under investigation, and we hope to determine precisely what the additional CF mutations are in our population.

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