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Transmission ratio distortion and maternal effects confound the analysis of modulators of cystic fibrosis disease severity on 19q13

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Two entities localised within in a 5 Mb interval on 19q13, that is the transforming growth factor β 1 (*TGFβ1*) and the cystic fibrosis modifier 1, have been reported to modulate disease severity of cystic fibrosis (CF), albeit the designation of the risk allele for *TGFβ1* differs between studies. We have analysed genotyping data at seven microsatellite loci and four single nucleotide polymorphisms targeting the 19q13 area from 37 nuclear CF families with two affected offspring exhibiting extreme clinical phenotypes for indicators of transmission-ration distortion, maternal genetic or maternal non-genetic effects. Evidence for a transmission-ratio distortion was obtained at D19S112 ($P = 0.0304$) near the recently characterised myotonic dystrophy locus myotonic dystrophy protein kinase (*DMPK*). Maternal and paternal genotype distributions were significantly different at rs1982073 (Leu10Pro at *TGFβ1*) whereby all CF sibs heterozygous at rs1982073 inherited the Leu10 allele from their mother ($P = 0.000132$) in our sibling panel. To ask whether the improved survival in CF over the last decades has any influence on *TGFβ1* allele frequencies, we analysed unrelated F508del homozygotes who were stratified by birth cohort. Sensitivity with respect to the survivor bias was reflected by significantly higher incidence of mild cystic fibrosis transmembrane conductance regulator mutation genotypes in the early born patient cohort ($P = 0.0169$), and an allelic imbalance was also observed at *TGFβ1* ($P = 0.0664$). In conclusion, the role of *TGFβ1* as a CF modulator, suggested from studies with a case–control setting, needs to be interpreted with caution unless family-based analysis is carried out to identify parental genetic and non-genetic effects.
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Introduction

The variability of the clinical course of cystic fibrosis (CF) suggests that factors other than the disease-causing cystic fibrosis transmembrane conductance regulator (*CFTR*) gene mutations shape the patients' phenotype.[1] In addition to the well-characterised role of the *CFTR* mutation genotype and environmental factors, modulating genes are considered in CF by an increasing number of studies.[2]

Two entities on 19q13, that is the gene encoding the transforming growth factor β 1 (*TGFβ1*) and the still undetermined gene mapped as cystic fibrosis modifier 1 (*CFM1*) in 1999, have been implied as modulators on CF pulmonary disease severity[3,4] and the CF endophenotype meconium ileus.[5]

The *European CF Twin and Sibling Study* applies the concept of informative patient pairs with extreme clinical phenotypes to identify and map CF modulators.[6–8]

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Recently, we have reported on the association of D19S197, but not of *TGFβ1* or the *CFM1*-related marker D19S112 with the CF disease phenotype.[9] Here, we have analysed our genotype data for indicators of transmission-ratio distortion, imprinting, maternal genetic or maternal non-genetic effects mediated by elements on 19q13.

Patients and methods

The European CF twin and sibling study patient panel
As described in detail previously,[6] we have recruited CF twin and sibling pairs and their parents from central Europe. For the identification of CF modulators, F508del-*CFTR* homozygous dizygous patient pairs with concordant mild disease, concordant severe disease and discordant pairs were selected.[6] In total, 37 nuclear families with contrasting phenotypes were enrolled for genotyping whereby parental DNA was obtained in 32 families.[8]

Unrelated F508del homozygotes stratified for year of birth
The long-term prognosis and survival has improved considerably over the last decades. In order to ask whether this manifests in the CF population at the typed markers, we have retrospectively recruited CF patients born in 1959–1967 or 1970–1975 who were enrolled for *CFTR* mutation analysis at the CF clinic in Hannover in 1989–1994. The *CFTR* mutation genotype was resolved for 130 patients, allowing to assess the proportions of mild and severe *CFTR* mutation genotypes by birth cohort. Mild *CFTR* mutation genotypes, associated with pancreatic sufficiency, better lung function and better survival,[10] were assigned in accordance with the European Epidemiologic Registry of CF.[10]

The study was approved by the local medical ethics committee. DNA of 21 F508del homozygotes born 1959–1967 and of 49 patients born 1970–1975 was available or could be rescued by whole genome amplification using the GenomiPhi-System (GE Healthcare).

Genetic markers
The European CF twins and sibs with extreme clinical phenotypes have been genotyped at seven microsatellites and four single nucleotide polymorphisms (SNPs) on 19q13 (Table 1). SNPs were analysed by PCR/RFLP and microsatellite genotypes were ascertained by direct blotting electrophoresis (GATC, Konstanz) as described elsewhere.[11] All technical details are specified within the supplement.

To allow the identification of haplotype blocks and correspondingly enable a correction for multiple testing within sets of linked markers, pairwise marker linkage disequilibrium (LD) was judged both on the basis of Lewontins D′ measure[12] as well as on the signicance of the χ^2 measure as described in detail previously.[9] As

expected, the two *TGFβ1* markers rs1982073 and rs1800469 were in LD defining block B (Table 1). Furthermore, two SNPs and two microsatellites spanning a 100 000 bp area from rs1126454 to rs4802129 were observed as block C on the present low-density map.

Data evaluation
As described in detail elsewhere,[9] nuclear families were analyzed with the Monte Carlo simulation based association test described by Knapp and Becker,[13] which can be viewed as an extension of the transmission–disequilibrium test[14] to both nuclear families with more than one affected child and to haplotypes.

Furthermore, we tested for parent-of-origin effects using the HAP-PAT introduced by Becker *et al*.[15] This test considers the parental origin of the alleles of heterozygous affected children. As shown by Weinberg,[16] this yields a very powerful test for imprinting and assocation. Significant results obtained with the HAP-PAT give evidence either for imprinting effects or for an effect of the maternal genotype on the child's phenotype. To distinguish the two possibilities, we also compared paternal and maternal genotypes using the case–control method described by Becker *et al*.[17]

All computations, that is the extended transmission-disequilibrium test, the parental asymmetry test HAP-PAT and the case–control comparisons, were carried out using the FAMHAP software package.[18]

Genotype and allele distribution among unrelated F508del homozygotes stratified for birth cohort were compared by permutation analysis through Monte Carlo simulation.[19]

Results
Analysis of all nuclear families pooled, irrespective of their phenotype by transmission–disequilibrium test did not yield a significant result at all loci tested, irrespective of whether both parental transmissions or only the paternal transmissions or only the maternal transmissions were taken into account (data not shown). In contrast, the parental origin of alleles was different among heterozygous offspring at *TGFβ1* (block B; $P_{corr} = 0.000145$; Table 1a and b) and marker D19S112 ($P = 0.0304$; Table 1a). Surprisingly, we observed that all heterozygous siblings inherited allele 2 from their mother at rs1982073 (Table 1b). Comparing maternal and paternal genotype frequencies, a significant difference was observed at *TGFβ1* (block B; $P_{corr} = 0.0339$; Table 1a and c).

To ask whether the improved survival in CF over the last decades has any influence on *TGFβ1* allele frequencies, we analysed unrelated F508del homozygotes from the CF clinic in Hannover who were stratified by birth cohort. Sensitivity with respect to the survivor bias is reflected by significantly higher incidence of mild *CFTR* mutation

Table 1a Comparison of parental origin of alleles in heterozygous offspring and parental genotype frequencies in 37 F508del homozygous CF sib pair families

Marker	Position on Chr19	Block	Parental asymmetry test HAP-PAT [a]		Comparison of maternal and paternal genotypes [b]	
			Uncorrected single locus P-value	Corrected P-value for block	Uncorrected single locus P-value	Corrected P-value for block
D19S400	46219373	A	0.5401	0.5401	0.3445	0.3445
rs1982073[c]	46550761	B	0.000132	0.000145	0.0123	0.0339
rs1800469	46552136	B	0.0325		0.3326	
rs1126454	46824154	C	0.1855	0.4444	0.1546	0.3531
D19S197	46824244	C	0.3343		0.6772	
CCSat1	46886606	C	0.6281		0.6830	
rs4802129	46933285	C	0.1740		0.2487	
CCSat3	47305349	D	0.2489	0.2489	0.3324	0.3324
CCSat6	47793292	E	0.2341	0.2341	0.4749	0.4749
PSGSat	48499978	F	0.7368	0.7368	0.3354	0.3354
D19S112	51070821	G	0.0304	0.0304	0.1487	0.1487

[a]The parental asymmetry test HAP-PAT analyzes the origin of alleles in nuclear families with heterozygous offspring. Primary genotyping data for rs1982073 is given in Table 1b.
[b]Primary data for rs1982073 is given in Table 1c.
[c]SNP rs1982073 corresponds to the mis-sense variant Leu10Pro in *TGFβ1*.

Table 1b Asymmetry of parental transmissions to heterozygous CF siblings at rs1982073

	Total no. of families with heterozygous offspring	No. of families with one heterozygous sibling	No. of families with two heterozygous siblings	Total number of heterozygous siblings
Observed maternal transmissions of allele 2[a] at rs1982073	15	9	6[b]	21
Observed paternal transmissions of allele 2[a] at rs1982073	0	0	0	0
Non-informative phase or no parental DNA available	6	1[c]	5[d]	11
Total	21[e]	10	11	32

[a]rs1982073 allele 2, denoting the presence of restriction site for BstAPI as assessed by PCR-RFLP test, corresponds to Leu10 in *TGFβ1*.
[b]The maternal transmission of allele 2 was observed for 12 siblings in these six families.
[c]For this family, DNA was available for one parent only who carries the heterozygous genotype.
[d]For three of these families, no parental DNA was available. In one family, both parents are heterozygous and in one family, DNA was available for one parent only who carries the heterozygous genotype.
[e]Out of the 37 families, 16 sib pairs were homozygous at rs1982073 who are not considered by the parental asymmetry test.[15,16]

Table 1c Parental genotype distribution at rs1982073

		Father n (frequency)	Mother n (frequency)
Genotype at rs1982073[a]			
	1–1	9	4
	1–2	14	10
	2–2	5	15
Total:		28	29
Alleles at rs1982073[a]			
	1	32 (0.57)	18 (0.31)
	2	24 (0.43)	40 (0.69)

[a]1982073 allele 2, denoting presence of restriction site for BstAPI as assessed by PCR-RFLP test, corresponds to Leu10 in *TGFβ1*.

genotypes in the early born patient cohort ($P = 0.0169$; Table 2a). Among the subsets of F508del homozygous patients from these birth cohorts, allele frequencies at rs1982073 were dissimilar ($P = 0.0664$; Table 2b).

Discussion

TGFβ1, displaying a leucine to proline exchange at codon 10 in the human population, has been identified as a modifier among F508del-*CFTR* homozygotes by two independent studies.[3,4] However, the authors disagree on the designation of the risk allele. Arkwright *et al*[3] describe the *TGFβ1* variant Leu10 as a risk allele for accelerated decline of pulmonary function with age among 171 CF patients from the North West region of the United Kingdom. In contrast, Drumm *et al*[20] have reported an elevated frequency of Pro10 *TGFβ1* alleles among more than 800 patients with a severe pulmonary phenotype recruited from 44 North American CF clinics, indicating that the *TGFβ1* risk allele is Pro10 in their patient panel. In conclusion, the data concerning the role of *TGFβ1* in CF is at best contradictory at the moment even though the functional consequences of Pro10, resulting in lower circulating levels

of the anti-inflammatory cytokine in serum, are well characterised.

The failure to replicate a finding in an association study can be explained by numerous reasons whereby false-negative results are usually attributed to a lack of power of one study. Sadly, this frequently quoted argument ignores the prerequisite for assuming two similar outcomes, namely that the two patient populations under study

are comparable. In the context of CF, the continuously changing symptomatic treatment and its consequences on manifestation of disease and improvement of survival will jeopardise any attempts to identify clinically relevant genetic modifiers, unless one thoroughly controls during recruitment of the study cohort for any bias of patient history introduced by date of birth and the previous quality of care. Such a confounding survivor effect is suggested by findings on the Leu10Pro polymorphism (rs1982073) in *TGFβ1* in our local CF population (see Table 2b).

In our set of European F508del homozygous CF twins and siblings, comparing concordant mildly and concordant severely affected sib pairs from matched birth cohorts,[8] no association of *TGFβ1* markers with disease severity was observed.[9] However, maternal and paternal genotype distributions were significantly different at rs1982073 (Leu10Pro at *TGFβ1*) whereby homozygosity for allele Leu10 was elevated among maternal genotypes and all CF sibs heterozygous at rs1982073 inherited the Leu10 allele from their mother ($P = 0.000132$, Table 1), demonstrating that maternal effects outweigh the inherited genetic predisposition at *TGFβ1*. In other words, the influence of the maternal genotype – as outlined below,

Table 2a Distribution of mild and severe *CFTR* mutation genotypes among two CF cohorts recruited in the early 1990 s and stratified for contrasting year of birth

Birth cohort	CFTR mutation genotype	No. of patients (frequency)
1959–1967	Severe[a]	37 (84%)
	Mild[b]	7 (16%)
1970–1975	Severe[a]	83 (97%)
	Mild[b]	3 (3%)

Patients carry two severe *CFTR* mutations according to the classification by the European Epidemiologic Registry of Cystic Fibrosis.[10]
Patients carry one mild *CFTR* mutation according to the classification by the European Epidemiologic Registry of Cystic Fibrosis.[10]
[a,b]Comparison of observed number of mild and severe genotypes: $P = 0.0169$.

Table 2b Allele distribution at rs1982073 among unrelated F508del homozygotes from contrasting birth cohorts

Birth cohort	CFTR mutation genotype	No. of patients [b]	rs1982073[a] Allele 1[c] n (frequency)	Allele 2[d] n (frequency)
1959–1967	F508del/F508del	21	7 (0.17)	35 (0.83)
1970–1975	F508del/F508del	49	32 (0.33)	66 (0.67)

[a]Comparison of allele distribution at rs1982073: $P = 0.0664$.
[b]Subset of patients with severe *CFTR* mutation genotype from Table 2a.
[c]rs1982073 allele 1, denoting the absence of restriction site for BstAPI as assessed by PCR-RFLP test, corresponds to Pro10 in *TGFβ1*.
[d]rs1982073 allele 2, denoting the presence of restriction site for BstAPI as assessed by PCR-RFLP test, corresponds to Leu10 in *TGFβ1*.
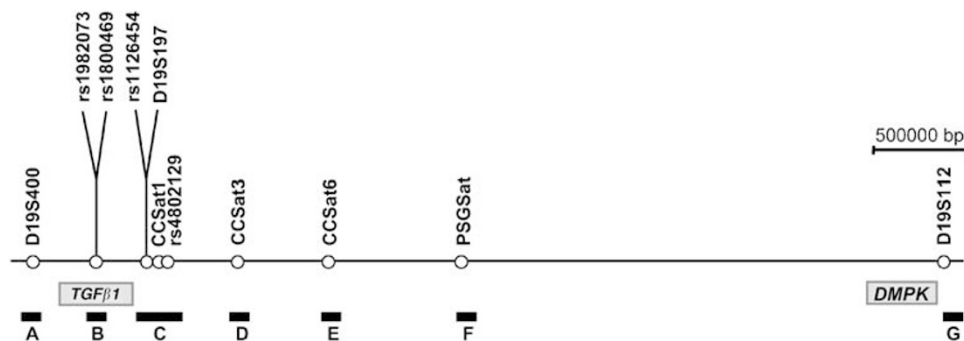

Figure 1 Physical map of the analysed area on 19q13. The map is drawn to scale representing physical distances on NT_011109 for markers D19S400, rs1982073/rs1800469, rs1126454/D19S197, CCSat1, rs4802129, CCSat3, CCSat6 and PSGSat and D19S112. The distance between the two *TGFβ1* SNPs rs1982073 (Leu10Pro) and rs1800469 (C-509 T) is 1375 bp. The SNP rs1126454 is localised in a distance of 90 bp to the microsatellite D19S197. The position of *TGFβ1*, published as a CF modulator by Arkwright *et al*[3] and by Drumm *et al*[4] with incompatible results with respect to the designation of the risk allele, is indicated below the physical map. *DMPK* is the myotonic dystrophy locus for which Dean *et al*[21] have described a transmission ratio distortion in human preimplantation embryos. For the sake of clarity, the genes *TGFβ1* and *DMPK1* are not drawn to scale. D19S112, reported to detect the cystic fibrosis modulator 1 by Zielenski *et al*[5], is located in a distance of 100 kb to *DMPK*. Capital letters below the bold bars correspond to the haplotype blocks reported upon in Table 1a.

presumably mediated by TGFβ1 supply to the offspring in human milk in early postnatal life – exceeds the life-long effect of variants of the anti-inflammatory, albeit profi-brotic cytokine mediated by the child's genotype itself. Moreover, evidence for a transmission-ratio distortion at D19S112 was observed ($P = 0.0304$, Table 1), presumably mediated by the myotonic dystrophy locus *DMPK* which is located 100 000 bp upstream of D19S112. A transmission-ratio distortion manifesting in human preimplantion embryos has recently been described for *DMPK*.[21]

Maternal TGFβ1, the major anti-inflammatory cytokine in human milk provided by breast-feeding to the infant, is known to protect against gut inflammation[22] and infant wheezing.[23] Long-term exclusive breast feeding was shown to attenuate disease severity in CF,[24] and consequently, the impact of maternal TGFβ1 in CF may be more prominent in patients with late diagnosis and/or delayed onset of therapeutic intervention. In this context, the interplay between exogenous maternal supply and endogenous production of TGFβ1 may deserve further investigation.

The CF modulator indicated by the previously described transmission disequilibrium among discordant pairs at D19S197[9] was confirmed with the present marker set as mildly and severely affected CF sib pairs displayed different allele distributions at rs4802129 ($P = 0.0161$; for uncorrected single locus; data not shown), indicating that the block C, encompassing the *CEACAM* gene cluster, harbours a genetic variant that shapes the clinical course of CF. Although the identity of the modulator cannot be resolved on our current low-density map, further investigation and high-resolution fine mapping of block C among CF twins and sibs is underway to identify the genetic entity on 19q13 that has an impact on CF disease severity (Figure 1).

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