The 8.1 ancestral MHC haplotype is associated with delayed onset of colonization in cystic fibrosis

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Abstract

Major cause of death in patients with cystic fibrosis (CF) is colonization with Staphylococcus aureus and Pseudomonas aeruginosa. The wide phenotypic variation in CF patients suggests that genes other than the cystic fibrosis transmembrane conductance regulator (CFTR) gene modify the disease. The 8.1 ancestral haplotype (8.1AH) in main histocompatibility complex is associated with alterations of the immune response. To study the influence of carriage of 8.1AH on frequency and onset of colonization in CF patients, DNA samples of 72 CF patients (39 homozygous and 33 heterozygous for ΔF508) were genotyped for member alleles of the 8.1AH: HLA-DQB1*0201, HLA-DRB1*0301, receptor for advanced glycation end products (AGER) –429C, HSP70-2 –1267G (HSP70-2G) and tumor necrosis factor-α (TNF-α) –308A (TNF2). Colonization was verified by regular clinical and bacteriological screening. Frequency of colonization was significantly (P = 0.012) lower in the 8.1AH carriers; age, gender and ΔF508 genotype-adjusted odds ratio to be colonized of the carriers versus non-carriers was 0.112 (0.024–0.520). According to survival analysis, patients with 8.1AH had significantly (P < 0.0001) longer colonization-free period compared with non-carriers. Our novel observations demonstrate that the 8.1AH is associated with delayed onset of colonization in CF, presumably by influencing defense mechanisms against infections.

Introduction

Cystic fibrosis (CF), the most common lethal autosomal recessive genetic disorder in the Caucasian population, is caused by mutations in a 230-kb gene on chromosome 7 encoding a 1480 amino acid polypeptide, named cystic fibrosis transmembrane conductance regulator (CFTR), which functions as anion channel in epithelial membranes (1). The most frequent mutation is caused by deletion of phenylalanine at position 508 (ΔF508). The vast majority of CF patients suffer from chronic bacterial lung infection/inflammation caused most frequently by Staphylococcus aureus and/or Pseudomonas aeruginosa. Such infection is also called colonization and is responsible for the reduced life expectancy of CF patients (2). However, the wide phenotypic variation in patients homozygous for ΔF508 (3–8) suggests that genes other than CFTR, environmental factors or both modify development, progression and severity of the disease. Therefore, the onset and severity of pulmonary disease are not easily predicted (9, 10). Therefore, identification of factors that may modify the course
of CF is of utmost importance (1). Most of the candidate modifier genes that may affect the clinical course of CF, such as transforming growth factor β (11), mannose binding lectin (12, 13), nitric oxide synthase 1 (14) or genes localized in the major histocompatibility complex (MHC) (15, 16) and tumor necrosis factor-α (TNF-α) (3–8) play important roles in the innate or acquired immune response.

There is vast controversy in the role of several modifier gene candidates in CF, since correlations with the clinical status of CF patients remain doubtful. The TNF-α−308A (TNF2) allele is a good example for the conflicting data between pulmonary phenotype and disease severity. Hull and Thomson (6) found the TNF2 allele to be associated with a decrease in forced expiratory volume1. Other studies could not confirm association with any markers of disease severity (7, 8). In all of these cases, separate single-nucleotide polymorphisms (SNPs) were studied, without the context of a haplotype. Haplotype differences mean that discrepancies may arise from the differences in the distribution of certain alleles in different populations. In addition, the interaction of possible modifier genes with environmental factors such as exposure to tobacco smoke may also contribute to diversity in the clinical picture of the disease (17).

The term ‘ancestral haplotype’ defines highly conserved haplotype—blocks of highly conserved genomic sequences—derived from a common remote ancestor (18). One of these ancestral haplotypes (AHs), called 8.1AH is encoded in the MHC region on the short arm of chromosome 6 (19). 8.1AH Individuals have altered cytokine profile, increased TNF-α production, high titers of autoantibodies and circulating immune complexes, which are thought to be beneficial in response to infections. However, 8.1AH may lead to the development of autoimmune diseases as a long-term side effect (20, 21). The 8.1AH consists of, among others, the HLA-A1, B8, Cw7, DQB1*0201 (DQ2), DRB1*0301 (DR3), DQA1*0501, TNF2 and C4A*Q0 alleles (18, 20, 21). According to a study of Price et al. (18), receptor for advanced glycation end products (AGER) −429C and HSP70-2G (HSP70-2 −1267 G) alleles are candidate members of the 8.1AH.

We hypothesized that the 8.1AH has a role in chronic infections (colonization) in CF. Therefore, we determined HLA-DQB1, HLA-DRB1, TNF−α−308 G > A, AGER −429 T > C as well as HSP70-2 −1267 A > G polymorphisms in 72 CF patients (39 patients were homozygous and 33 patients were heterozygous for ΔF508) and TNF−α−308 G > A, AGER −429 T > C and HSP70-2 −1267 A > G polymorphisms in 139 healthy controls. All patients registered at seven Hungarian CF centers with available data about the onset of colonization were included in the study.

Methods
Study population
We have studied 39 CF patients, homozygous for ΔF508, aged 1–28 years (median age 11 years, 25–75%: 4–18 years), and 33 CF patients heterozygous for ΔF508, aged 3–35 years (median age 12 years, 25–75%: 6.5–17.5 years), attending seven Hungarian CF centers. In all 72 cases, diagnosis of CF has been proven both by typical clinical features, abnormal sweat test, and genetic tests, i.e. mutations in the CFTR gene.

Lung colonization with P. aeruginosa and S. aureus was investigated by routine microbiological methods collected at least once or twice a year during center visits. Colonization was defined from the first positive recorded culture provided if stayed positive subsequently. A patient with annual throat culture was considered non-colonized if such cultures were not persistently positive for a given pathogen. All colonized patients were colonized with P. aeruginosa and/or S. aureus, no colonization with Burkholderia, Stenotrophomonas, Achromobacter or Nontuberculous mycobacteria was detected in the studied population. All study patients were followed from at least the age of onset of colonization. Four patients were vaccinated against P. aeruginosa, none of them were colonized and none of them carried the 8.1AH. Treatment and care of CF patients are determined by national guidelines in every Hungarian CF center. Written consent was received from each patient for the use of their DNA samples in this study. The procedures followed were in accordance with the ethical standards of the Hungarian Ethical Committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 1983. Written informed consent was received from patients for the use of their DNA samples in this study. Deceased patients were not included in the study.

139 healthy persons (56 males, 83 females, 29.4 ± 3.9 years old) who volunteered for the study served as controls.

Genotyping methods
Genotyping of the following alleles, TNF−α−308 (22), AGER −429 (23) and HSP70−2 (24, 25) from MHC III region and HLA-DQB1 (Olerup SSP TM DQ kit, Olerup SSP AB, Saltsjöbaden, Sweden) and HLA-DRB1 (INNO-LIPA HLA-DRB1 Plus kit, Innogenetics, Ghent, Belgium) from the MHC II region was carried out by sequence-specific priming–PCR (HLA-DQB1, TNF−α−308), sequence-specific oligonucleotide probes–PCR (HLA-DRB1) and PCR–restriction fragment length polymorphism (AGER −429, HSP70−2) in the genomic DNA of CF patients. Carriage of the 8.1AH haplotype was considered when AGER −429C, HSP70−2G, TNF −308A, HLA-DRB1*0301 and HLA-DQB1*0201 were carried simultaneously.

Statistical analysis
For statistical evaluation, GraphPad Prism 4.00 for Windows software package was used (GraphPad Software, San Diego, CA, USA, http://www.graphpad.com). Significance was considered at P < 0.05. Power analysis was performed by using the GraphPad StatMate software (GraphPad Software). All differences found to be significant at the univariate analysis were checked by multivariate logistic regression models. Length of colonization-free period between groups of the patients was compared by survival curve analysis using the Mantel–Haenszel log rank test for comparison. Ages of patients were compared by Mann–Whitney test.

Results
Occurrence of the MHC III marker alleles and haplotypes of the 8.1AH in CF patients and normal population
The frequency of three 8.1AH marker alleles encoded in the MHC III (central region of MHC), TNF−308A (TNF2), HSP70-2

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1267G (HSP70-2G) and the AGER –429C was determined in 72 patients with CF. Since we have not determined the frequencies of the MHC II marker alleles [HLA-DQ2 and HLA-DR3 (17)] in our control group yet, we could not perform the comparison for these alleles (Table 1).

No significant differences between the whole group of CF patients and healthy controls were found in the frequency of either 8.1AH marker allele or haplotypes. The patients were divided into two groups, those who were colonized till the time of the study \( (n = 44) \) and those who were not \( (n = 28) \). Dramatic differences were found in the case of non-colonized patients: the frequency of all but one (HSP70-2G) alleles and all but one haplotypes tested was significantly higher among these patients than in healthy controls. The highest difference was found with the TNF2-HSP70-2-G-AGER –429C haplotype, it occurred more than three times more frequently among the non-colonized patients than among the controls. By contrast, the frequency of neither alleles nor haplotypes tested significantly differ between the groups of already colonized patients and healthy controls (Table 1).

Since the age of the non-colonized CF patients \( (9.8 \pm 6.2 \text{ years}) \) was substantially lower than that of the controls \( (29.4 \pm 3.9 \text{ years old}) \), we performed an age- and gender-adjusted multiple regression analysis, and found that the difference between the two groups in the TNF2-HSP70-2-G-AGER –429C haplotype remained significant \( (P = 0.031) \) after adjustment.

### CF population stratified according to carriers and non-carriers of the 8.1AH—modifier effect of the 8.1AH on colonization

The basic clinical characteristics of the study population are presented in Table 2. No significant difference according to the carriage of the 8.1AH was found in gender, median age and proportion of ΔF508 homozygotes and heterozygotes in the 72 CF patients. ΔF508 heterozygous patients carrying the 8.1AH were slightly but not significantly \( (P = 0.086) \) older than non-carriers.

By contrast, we found marked differences between carriers and non-carriers of the 8.1AH in the percentages of colonized and non-colonized patients as well as in the age at colonization (Table 2). The median age at colonization was significantly higher and the proportion of patients colonized with *P. aeruginosa* and/or *S. aureus* was significantly lower in the group of the 8.1AH carriers, as compared with non-carriers. One-quarter versus two-thirds, respectively, of 8.1AH carriers versus non-carriers were found to be colonized. According to power analysis, sample size was sufficient to detect a significant difference with 80% power. When only

### Table 1. Frequency of the MHC III marker alleles and haplotypes in CF patients and healthy controls

<table>
<thead>
<tr>
<th>Allele (haplotype)</th>
<th>Allele (haplotype) frequency</th>
<th>Healthy controls ((n = 139))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patients with CF</td>
<td></td>
</tr>
<tr>
<td></td>
<td>All patients ((n = 72))</td>
<td>Non-colonized patients ((n = 28))</td>
</tr>
<tr>
<td>TNF2 (−308A)</td>
<td>0.155</td>
<td>0.304***</td>
</tr>
<tr>
<td>HSP70-2−1267G (HSP70-2G)</td>
<td>0.420</td>
<td>0.519</td>
</tr>
<tr>
<td>AGER −429C (AGER-C)</td>
<td>0.181</td>
<td>0286</td>
</tr>
<tr>
<td>TNF2-HSP70-2G</td>
<td>0.140</td>
<td>0.259***</td>
</tr>
<tr>
<td>TNF2-AGER-C</td>
<td>0.104*</td>
<td>0.214**</td>
</tr>
<tr>
<td>HSP70-2G-AGER-C</td>
<td>0.160</td>
<td>0.259*</td>
</tr>
<tr>
<td>TNF2-HSP70-2G-AGER-C</td>
<td>0.104*</td>
<td>0.222***</td>
</tr>
</tbody>
</table>

Difference to the healthy controls (Fisher’s exact test), *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \).

### Table 2. Data of CF patients \((n = 72)\) stratified according to the 8.1AH

<table>
<thead>
<tr>
<th>8.1AH+ ((n = 14))</th>
<th>8.1AH− ((n = 58))</th>
<th>( P ) value(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/female</td>
<td>5/9</td>
<td>34/24</td>
</tr>
<tr>
<td>Age(^b) (years)</td>
<td>16.5 (8.3–20.5)</td>
<td>10 (4–17)</td>
</tr>
<tr>
<td>ΔF508 homozygote/heterozygote</td>
<td>5/9</td>
<td>34/24</td>
</tr>
<tr>
<td>Colonized with <em>Pseudomonas aeruginosa</em></td>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td>Colonized with <em>Staphylococcus aureus</em></td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td>Colonized with <em>P. aeruginosa</em> + <em>S. aureus</em></td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>Colonized total</td>
<td>4</td>
<td>40</td>
</tr>
<tr>
<td>Non-colonized</td>
<td>10</td>
<td>18</td>
</tr>
<tr>
<td>Age at onset of colonization(^c) (years)(^d)</td>
<td>3.0 (1.0–8.0)</td>
<td>14.0, 17.0</td>
</tr>
<tr>
<td>ΔF508 homozygotes</td>
<td>4.0 (3.0–10.0)</td>
<td>3.0, 19.0</td>
</tr>
<tr>
<td>ΔF508 heterozygotes</td>
<td>15.5 (5.4–17.8)</td>
<td>3.0 (0.8–8.0)</td>
</tr>
</tbody>
</table>

\(^a\)Number and median age of patients are shown. \(^b\)Mann–Whitney test or \( \chi^2 \) test. \(^c\)Median age (25–75% percentile). \(^d\)Only patients already colonized were included.
patients already colonized were included, the median length of colonization-free period was more than five times longer in 8.1AH carriers ($P = 0.036$) (Table 2).

These differences obtained at univariate analysis could be confirmed by multiple logistic regression analysis. In a multivariate logistic regression model, adjusted for age, gender and $\Delta F508$ genotype, odds ratio (95% confidence interval) to be colonized of the carriers versus non-carriers of the 8.1AH was 0.112 (0.024–0.520; $P = 0.005$) (Table 3).

The only CF patient who was homozygous for the 8.1AH got colonized at the age of 17, despite the fact that this patient was $\Delta F508$ homozygous.

**Comparison of the differences between the 8.1AH carriers and non-carriers by survival analysis**

The colonization-free periods in the groups were compared by survival analysis, Mantel–Haenszel log rank test. When both groups of CF patients were analyzed together, in carriers of the 8.1AH the difference in the length of colonization-free period was found to be highly significant ($P < 0.0001$) (Fig. 1a). In $\Delta F508$ homozygous CF patients who carried the 8.1AH, we observed significantly longer period free of colonization with *S. aureus* and/or *P. aeruginosa* ($P = 0.029$) compared with non-carriers (Fig. 1b). In patients heterozygous for $\Delta F508$, the length of colonization-free period was also significantly longer in carriers of the 8.1AH than in non-carriers ($P = 0.004$) (Fig. 1c). Curves ending vertically indicate that $\Delta F508$ homozygous patients, both carriers and non-carriers of the 8.1AH, get colonized by the time they reach the oldest age the curves indicate in Fig. 1(b). On the contrary, by curves ending horizontally, Fig. 1(a and c) reflects that in the overall CF population and in $\Delta F508$ heterozygotes, those at the oldest age indicated by the curves are not all colonized.

Most of the data published refer to CF patients with *P. aeruginosa* colonization. Therefore, in the next step, we restricted the analysis to patients who are colonized with *P. aeruginosa*. As colonization is a process, the first colonizing strain is not solely *P. aeruginosa*, but all patients in this group are positive for *P. aeruginosa*. A tendency similar to the previous ones was observed; the difference between the carriers and non-carriers of the 8.1AH was significant, too, when colonization-free period was compared in the 31 CF patients who were positive for *P. aeruginosa* ($P = 0.025$, data not shown).

**Table 3.** Strength of association with colonization of the 8.1AH in 72 CF patients, calculated by multivariate logistic regression analysis

<table>
<thead>
<tr>
<th></th>
<th>Odds ratio (95% confidence interval)</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unadjusted</td>
<td>0.180 (0.050–0.651)</td>
<td>0.009</td>
</tr>
<tr>
<td>Adjusted to age and gender</td>
<td>0.100 (0.022–0.455)</td>
<td>0.003</td>
</tr>
<tr>
<td>Adjusted to age, gender and $\Delta F508$ genotype</td>
<td>0.112 (0.024–0.520)</td>
<td>0.005</td>
</tr>
</tbody>
</table>

*a8.1AH carriers versus non-carriers.*

**Discussion**

The novel observation of the present study is that carriership of the 8.1AH is associated with low frequency and markedly delayed onset of colonization with both *S. aureus* and *P. aeruginosa* in CF patients. Interestingly, the one and only CF patient who was homozygous for the 8.1AH became colonized at the age of 17, despite the fact that this patient was $\Delta F508$ homozygous. No genetic factor which is thus strongly related to the time of colonization has been described so far. Recently De Rose et al. (26) reported on a related observation: they found homozygous R/R carriers of the Fc gamma receptor IIA gene associated with increased risk of infection by encapsulated organisms more frequently in CF patients with chronic *P. aeruginosa* infection than in uninfected patients or the general population; the differences were, however, much less.

**Fig. 1.** Colonization-free period in (a) CF patients homozygous and heterozygous for $\Delta F508$ ($n = 72$), (b) $\Delta F508$ homozygous CF patients ($n = 39$) and (c) $\Delta F508$ heterozygous CF patients ($n = 33$), carrying (solid line) and not carrying (broken line) the 8.1AH. A significant difference is present between the two groups of patients; (a) $P < 0.0001$, (b) $P = 0.029$, (c) $P = 0.004$ (Mantel–Haenszel log rank test).
significant \(P = 0.042\) than those with the 8.1AH in our present study.

Our present findings indicate that carriage of 8.1AH may influence the clinical course of CF, and determination of the 8.1AH could be used in clinical practice for predicting progression rate of CF patients. On the other hand, vaccination against \(P. aeruginosa\) was shown to prevent or to delay acquisition of bacteria, and to preserve lung function by, at least in part, induction of antibodies with higher binding affinity (27). Our observations may contribute to application of vaccines by categorizing CF patients into high-risk and low-risk groups in terms of infection, and may account for changes in therapy and immune prophylaxis. Due to the pathophysiology of the disease—the presence of thick mucus in the airways—CF patients are immunocompromised; thus, deviations in the genetically encoded features of the immune response may result in detectable differences concerning infections.

Host–pathogen interactions are crucial in the defense against infections. Though we cannot give a definite explanation for the delayed onset of colonization in the carriers of the 8.1AH, our present observation, however, is in line with several reported characteristics of the haplotype. Carriers of the 8.1AH have a genetically determined alteration in immune response; that is, this haplotype, besides an increased susceptibility to autoimmune diseases, is thought to confer resistance to infections (20, 21). The 8.1AH has been partly characterized by the presence of certain \(MHC\) class I and II alleles that, because of their unique role in antigen presentation and processing, were important targets of studies on modifier genes in CF. Since one of these putative alleles, \(TNF2\), is associated with the 8.1AH in only about 60% of the population, our findings may contribute to a better understanding of the conflicting results that were reported on the association between \(TNF2\) allele alone and markers of disease severity in CF patients (3–8). These data may also emphasize the importance of haplotype analysis rather than determination of SNPs as modifier factors, as previously suggested by others in the context of disease association studies (27–29). All known features of the 8.1AH, that is, increased \(TNF-\alpha\) production, deficient handling of circulating immune complexes and peculiarities in antigen presentation and processing and antibody production (18, 20, 21), may influence the effectiveness of the defense against different microorganisms. In this study, CF served as a model for increased susceptibility to infection. Thus, the data we have shown here for colonization in CF patients may be applicable for infectious complications in other diseases, such as sepsis or chronic obstructive pulmonary disease.

We are aware that our results need confirmation; further studies involving different countries are essential to test this hypothesis. However, if our present findings are reproducible, by the help of determining the carriage of the 8.1AH, CF patients could be divided into low-risk and high-risk groups, in terms of colonization. This could be useful for a better stratification in several immunological, microbiological and pharmaceutical studies in CF concerning infection, colonization as targets. As the geographical distribution of the \(\Delta F508\) allele shows similar pattern to that of the 8.1AH in Europe (30, 31), extended studies may have a chance to clarify whether during evolution the 8.1AH (as a selection advantage in carriers of the \(\Delta F508\) allele) could contribute to the maintenance of CF. In addition, findings of this study may have great relevance to newborn screening for CF.

**Acknowledgements**

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**Abbreviations**

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>AGER</td>
<td>receptor for advanced glycation end products</td>
</tr>
<tr>
<td>AH</td>
<td>ancestral haplotype</td>
</tr>
<tr>
<td>CF</td>
<td>cystic fibrosis</td>
</tr>
<tr>
<td>CFTR</td>
<td>cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>SNP</td>
<td>single-nucleotide polymorphism</td>
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<tr>
<td>TNF-(\alpha)</td>
<td>tumor necrosis factor-(\alpha)</td>
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</table>

**References**


