Haplotypes of the ApoA-I/C-III/A-IV Gene Cluster and Familial Combined Hyperlipidemia

Esa Tahvanainen, Päivi Pajukanta, Kimmo Porkka, Sari Nieminen, Liisa Ikävalko, Ilpo Nuotio, Marja-Riitta Taskinen, Leena Peltonen, Christian Ehnholm

Abstract—Familial combined hyperlipidemia (FCHL) is the most frequent familial lipoprotein disorder associated with premature coronary heart disease. However, no genetic defect(s) underlying FCHL has been identified. A linkage between FCHL and the apoA-I/C-III/A-IV gene cluster has been reported but not verified in other populations. A recent study identified FCHL susceptibility haplotypes at this gene cluster. To study whether such haplotypes are also associated with FCHL susceptibility in Finns, we studied 600 well-defined Finnish FCHL patients and their relatives belonging to 28 extended FCHL families by using haplotype, linkage, sib-pair, and linkage disequilibrium analyses. The genotypes of the MspI polymorphisms were associated with total serum cholesterol ($P<0.01$) and apoB ($P<0.05$) levels in spouses, which represent the general Finnish population. However, no evidence of direct involvement of any of these loci or their specific haplotypes in the expression of FCHL in the Finnish FCHL families was found. (Arterioscler Thromb Vasc Biol. 1998;18:1810-1817.)

Key Words: apoA-I ■ apoC-III ■ apoA-IV ■ familial combined hyperlipidemia ■ coronary heart disease

Familial combined hyperlipidemia (FCHL), originally described by 3 independent groups, is characterized by an elevation of cholesterol and/or triglyceride levels. In Western societies, the condition is found in 1% to 2% of the population and in ≈10% of survivors of myocardial infarction, designating FCHL as one of the most frequent familial dyslipidemias associated with premature coronary heart disease (CHD). In affected subjects, a common characteristic is an increase in small, dense LDL particles. A consistent metabolic finding in FCHL is an increased apoB plasma level, which is due to either increased production or lowered clearance of apoB-containing lipoproteins.

Association between the apoA-I/C-III/A-IV gene cluster on chromosome 11 and FCHL has been the subject of several studies. An association between FCHL and an $XmnI$ restriction fragment polymorphism in the 5’-flanking region of the apoA-I gene was reported, and the finding was later confirmed by linkage (logarithm of the odds [lod] score, 6.86) without recombinants in 7 families. Further evidence for an association between this gene cluster and FCHL has been provided by other groups, but the findings have also been challenged, and the positive linkage result has not been confirmed. An explanation for this controversy may be provided by a recent study, in which the contribution of the apoA-I/C-III/A-IV gene cluster is presented as an epistatic interaction between different haplotypes, a finding that may also partially explain the paradigm of the complex and varying phenotypes of FCHL. A specific combination of haplotypes (1-1-2/2-2-1, “the high-risk haplotype combination”) derived from polymorphic $XmnI$ and $MspI$ sites in the 5’-flanking region of the apoA-I gene and the $SsrI$ site in the 3’-untranslated region of the apoC-III gene was reported to be more frequent in hyperlipidemic patients (frequency, 0.06) than in normolipidemic relatives (frequency, 0.03, $P<0.05$) or spouses (frequency, 0.005, $P=0.01$). The aim of the current study was to test whether these specific haplotypes are also associated with FCHL in Finnish FCHL families.

Methods

Study Subjects

The collection of carefully documented pedigrees with several affected individuals has been reported. The study design was approved by the ethics committees of the participating centers, and each subject gave informed consent before participation in the study. A total of 28 pedigrees with 600 subjects were included in the study in 3 phases. In the first phase, probands fulfilling the following criteria were selected from hospital records: age 30 to 55 years for men and 30 to 65 years for women; at least a 50% stenosis in 1 or more coronary arteries confirmed by coronary angiography; and serum total cholesterol and/or serum triglycerides higher or equal to that of the age- and sex-specific 90th percentile. Exclusion criteria for the proband were type 1 diabetes, hypothyreosis, and hepatic or renal disease. In the second phase, the proband and all first-degree relatives were examined. Families with several affected individuals and at least 2 different lipid phenotypes, of which 1 was the...
combined lipid phenotype IIB in the proband or his/her first-degree relative, were included in the third phase, for which all accessible relatives and their spouses were examined. The characteristics of the study population are shown in Table 1.

**Biomedical Analyses**

The biochemical analyses and baseline data have been described in detail. In short, for each family member over the age of 5, blood was drawn after an overnight fast for the measurement of serum lipids and DNA isolation. Subjects using lipid-lowering agents were asked to interrupt their medication for 4 weeks before blood sampling. Serum samples were stored frozen at -70°C until analyzed. Serum total cholesterol and triglycerides were determined with automated enzymatic methods. HDL cholesterol was determined as described. Familial hypercholesterolemia was excluded from each pedigree by determining the LDL receptor status of the proband by using the lymphocyte culture method. None of the study subjects had tendon xanthomas.

**Genotyping**

DNA was isolated by a phenol-chloroform extraction method. Three polymorphisms, *Xmn*I, *Msp*I, and *Sst*I, at nucleotide positions -2500CTI and -78GaA of the apoA-I gene and at 3175G/C of the apoC-III gene, were determined. In the solid-phase minisequencing method used, variable nucleotides are identified by a single nucleotide primer extension reaction catalyzed by DNA polymerase from a polymerase chain reaction (PCR) product on a solid support. Three different primers were used to study each polymorphism: each DNA fragment, containing a nucleotide to be sequenced, was first amplified by PCR by using a pair of primers, and then the product was analyzed by a detection primer required for minisequencing method. With this coding scheme, allele 1 was found to be the most common allele for all polymorphisms.

**Linkage and Sib-Pair Analyses**

The linkage analyses were computed by using LINKAGE and FASTLINK program versions 5.1 and 2.3, respectively. The possible heterogeneity of FCHL was tested, and an affected sib-pair study on nuclear families was done by the programs HOMOG and SIBPAIR. The linkage analyses were calculated by using both a dominant and a recessive mode of inheritance. Based on the estimated FCHL prevalence, population gene frequencies of 0.004 and 0.089 for the dominant and recessive models, respectively, were used. Frequency of phenocopies was estimated to be 0.005, and penetrance, 0.90 in the calculations. To determine individuals status, the age- and sex-specific 90th percentiles of lipids derived from FINMONICA, a large population-based survey done in 1992, were used for both probands and family members. The fractile cut points for subjects under the age of 25 were derived from the Cardiovascular Risk Factors in Young Finns study. To reduce problems caused by unknown penetrance, subjects were coded as affected in linkage, sib-pair, haplotype relative risk (HRR), and tests of transmission disequilibrium (TDT) analyses if they had cholesterol and/or triglycerides >90th age- and sex-specific percentiles; healthy, if they had both cholesterol and triglycerides <30th age- and sex-specific lipid percentiles; and unknown, if they had lipid values between the 30th and 90th age- and sex-specific percentiles. If a spouse of a family member in the second generation or any of the 28 families was also affected according to the lipid criteria, the offspring of these couples were not included in the calculations to avoid bilinear introduction of the trait. Allele frequencies of 0.214, 0.198, and 0.089 for the dominant and recessive models, respectively, were used in the linkage analyses. Power calculations for linkage analysis were done by using the SLEINK program. With the use of a dominant model with linkage parameters given above and a marker with 4 alleles with equal frequencies, the average simulated lod score was 20.30 (θ=0.00); for a recessive model, the lod was 11.33 (θ=0.00).

**TDT, HRR, and Linkage Disequilibrium (LD)**

TDTs and HRR analyses were done through ANALYZE, an accessory program to the LINKAGE package, which simplifies the use of programs TDTLIKE and HRLAMB for this purpose. The HRLAMB program selects the first affected individual from each pedigree for which both parents are typed and then tests the nonrandom segregation of the transmitted and untransmitted alleles by a likelihood ratio test. The likelihood ratio–based TDT considers all the alleles jointly and tests whether 1 of them is transmitted from heterozygous parents preferentially to affected offspring. LD analyses between
polymorphisms were estimated by a permutation procedure, and Hardy-Weinberg equilibrium of genotypes and haplotypes was tested by an exact test; both methods are part of the program package ARLEQUIN. Haplotypes were determined by combining the use of haplotype estimation algorithms of the GENEHUNTER program package, version 1.0, and inspection of the pedigrees. The nonrandom distribution of alleles between probands and spouses was tested by Fisher’s exact test; the distribution of genotypes and haplotypes by a χ² test; and haplotype combinations by an exact test analogous to Fisher’s exact test extended to a 2×k contingency table, where k is the number of haplotype combinations.

Logistic Regression Analyses and ANOVAs

To study the effects of specific haplotype combinations in the pedigrees, we used logistic regression modeling and variance analysis. Subjects were coded as hyperlipidemic if they had cholesterol and/or triglycerides >90th age- and sex-specific percentiles. Triglyceride values were logarithmically transformed (base e) in all analyses. The regression analyses and ANOVAs were done with the Statistical Package for the Social Sciences (SPSS Inc), version 6.1.3. In the logistic regression analysis, the association of the haplotype combinations with FCHL phenotype was tested after adjusting for sibship. Two alternative models were tested for each haplotype combination and for all haplotype combinations together: a model in which only the sibship was known for each individual and a second model in which haplotype combinations were added in the model. The statistical significance of the comparison between these 2 models was tested on the basis of the change in log likelihood. The effects of the haplotype combinations on triglyceride, cholesterol, and apoB levels were studied by variance analysis adjusted for sibship, age, sex, and body mass index. In addition, the contribution of genotypes of each polymorphism and of haplotype combinations on serum total cholesterol, triglyceride, and apoB levels was studied in spouses by ANOVA.

Results

Linkage and Sib-Pair Analyses

The results of 2-point linkage analyses between FCHL phenotype and the polymorphisms remained nonsignificant with the use of both a dominant and a recessive model. The maximum lod scores of SstI, MspI, and XmnI polymorphisms with the dominant mode of inheritance were 0.00 (0.50), 0.02 (0.50), and 0.29 (0.22), respectively. (The recombination fractions are given in parentheses.) With the recessive model, the maximum lod scores were 0.08 (0.32), 0.00 (0.50), and 0.04 (0.36), with the same order of markers. In multipoint analyses with MLINK, the maximum lod scores were 0.27 (0.30) with a dominant mode of inheritance and 0.29 (0.28) with a recessive model. In the nonparametric affected sib-pair analyses, there was no excess allele sharing detectable. There was no significant heterogeneity detectable by the HOMOG program.

TDT, HRR, and LD Analyses

No significant differences in the frequencies of alleles, genotypes, haplotypes, or haplotype combinations were observed between probands and spouses. The frequencies are also given for family members divided according to hyperlipidemic or normolipidemic status, although the statistical comparisons between these latter groups cannot be done by direct comparisons between frequencies because they do not represent independent study subjects (Tables 2 and 3). The distribution of single-locus genotypes, haplotypes, and haplotype combinations did not differ significantly from Hardy-Weinberg equilibrium determined from total allele and haplotype frequencies, with 1 exception: in spouses, the frequencies of haplotypes deviated from those estimated from allele frequencies (P < 0.05). That finding was due to the fact that 2 polymorphisms, XmnI and MspI, were in LD (D′ = 0.52 for rare alleles, P < 0.05). No significant LD between FCHL

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Probands</th>
<th>Hyperlipidemics</th>
<th>Normolipidemics</th>
<th>Spouses</th>
</tr>
</thead>
<tbody>
<tr>
<td>111</td>
<td>0.50</td>
<td>0.53</td>
<td>0.64</td>
<td>0.61</td>
</tr>
<tr>
<td>112</td>
<td>0.19</td>
<td>0.21</td>
<td>0.13</td>
<td>0.17</td>
</tr>
<tr>
<td>121</td>
<td>0.04</td>
<td>0.07</td>
<td>0.06</td>
<td>0.04</td>
</tr>
<tr>
<td>122</td>
<td>0.02</td>
<td>0.01</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>211</td>
<td>0.07</td>
<td>0.04</td>
<td>0.04</td>
<td>0.09</td>
</tr>
<tr>
<td>212</td>
<td>0.02</td>
<td>0.02</td>
<td>0.00</td>
<td>0.01</td>
</tr>
<tr>
<td>221</td>
<td>0.15</td>
<td>0.13</td>
<td>0.11</td>
<td>0.07</td>
</tr>
<tr>
<td>222</td>
<td>0.02</td>
<td>0.01</td>
<td>0.02</td>
<td>0.01</td>
</tr>
</tbody>
</table>

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TABLE 4. The Mean Values of Serum Total Cholesterol, Triglyceride, and ApoB Levels in Spouses According to XmnI, MspI, and SstI Genotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Cholesterol, mmol/L</th>
<th>TG, mmol/L</th>
<th>ApoB, mg/100 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD (n)</td>
<td>Mean ± SD (n)</td>
<td>Mean ± SD (n)</td>
</tr>
<tr>
<td>XmnI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/1</td>
<td>5.43 ± 1.00 (79)</td>
<td>1.31 ± 0.62 (79)</td>
<td>96.0 ± 0.23 (79)</td>
</tr>
<tr>
<td>1/2</td>
<td>5.56 ± 1.13 (47)</td>
<td>1.38 ± 0.69 (47)</td>
<td>96.0 ± 0.31 (48)</td>
</tr>
<tr>
<td>2/2</td>
<td>5.55 ± 0.78 (2)</td>
<td>1.75 ± 1.48 (2)</td>
<td>68.0 ± 0.1 (1)</td>
</tr>
<tr>
<td>MspI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/1</td>
<td>5.29 ± 0.91 (91)</td>
<td>1.28 ± 0.67 (91)</td>
<td>91.7 ± 0.18 (91)</td>
</tr>
<tr>
<td>1/2</td>
<td>5.90 ± 1.21 (38)</td>
<td>1.44 ± 0.55 (38)</td>
<td>104.8 ± 0.29 (39)</td>
</tr>
<tr>
<td>2/2</td>
<td>5.55 ± 0.78 (2)</td>
<td>1.75 ± 1.48 (2)</td>
<td>68.0 ± 0.0 (1)</td>
</tr>
<tr>
<td>SstI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/1</td>
<td>5.43 ± 0.96 (69)</td>
<td>1.27 ± 0.59 (69)</td>
<td>93.1 ± 0.23 (69)</td>
</tr>
<tr>
<td>1/2</td>
<td>5.42 ± 1.09 (58)</td>
<td>1.33 ± 0.67 (58)</td>
<td>96.0 ± 0.29 (59)</td>
</tr>
<tr>
<td>2/2</td>
<td>7.05 ± 0.00 (1)</td>
<td>2.20 ± 0.00 (1)</td>
<td>151.5 ± 0.0 (1)</td>
</tr>
</tbody>
</table>

TG indicates triglyceride.

*P < 0.01. Covariates were age, sex, and BMI.
†ANOVA P < 0.05.

Discussion

Originally based on family inspection, analysis of the number of affected sibs, and the distribution of adjusted lipid values, it was suggested that FCHL constitutes an autosomal dominant trait.1,2 Segregation analyses have supported major gene effects on triglyceride levels, apoB levels, and LDL particle size distribution in affected families.3,4 The dominant model of inheritance is supported by a recent finding of linkage5 to chromosome 1q21-q23 in Finnish pedigrees. The linkage of a syntenic locus with FCHL has also been shown in a mutant mouse strain.6 Although this finding of linkage seems now very important, it has been noted several times in the course of research on FCHL that it is heterogeneous and also that in individual families, several genes can be involved in the expression of the FCHL phenotype. It is thus plausible that there are still other gene defects associated with FCHL in Finnish pedigrees. In fact, due to the broad definition of FCHL, defects causing FCHL may be found at any step along the metabolic pathways of apoB-containing lipoproteins; from the synthesis of VLDL and chylomicrons to the clearance of their end products, chylomicron remnants, and LDL from plasma.

Widely studied candidate genes of FCHL include the major genes affecting the metabolism of apoB-containing lipoproteins. The apoB gene locus does not contribute significantly to the development of FCHL, but whether the genes regulating its expression might contribute to the FCHL phenotype is not known.7 Mutations in the lipoprotein lipase gene and reduced postheparin plasma lipoprotein lipase activity have been found in FCHL patients in some studies,8,9 but not in all.17 The LDL receptor would be an obvious candidate gene for FCHL, but its defects cause a different phenotype and disease.10,11 Despite this, another still-unknown gene in close proximity to the LDL receptor gene may be associated with the small, dense LDL phenotype.12 Likewise, lipoprotein modulators like cholesterol ester transfer protein,9,10 lipoprotein(a),11 hormone-sensitive lipase,12 and hepatic lipase12 are potential candidates. In Finnish FCHL families, there was no evidence of linkage between FCHL and lipolytic enzymes.14,15 Defects in the insulin signaling pathway15 or in an even more potent adipin acylation stimulating protein signaling pathway are intensively studied candidate genes for FCHL. Adipsin acylation stimulating protein, also called basic protein I, is a fragment of the third component of plasma complement (C3a-des-Arg) and a strong modulator of triglycer-
Other cellular candidate genes include basic protein II, which affects cholesterol ester formation in the liver, and microsomal triglyceride transfer protein, which catalyzes the transport of triglyceride, cholesteryl ester, and phospholipid between phospholipid surfaces. One of the most important candidate loci of FCHL, and also the subject of the current study, is the apoA-I/C-III/A-IV gene cluster on chromosome 11. Taking into consideration previous partially contradictory results and the recent findings of complex genetic contribution of specific haplotypes, we considered it important to test the involvement of the apoA-I/C-III/A-IV gene cluster in the expression of the FCHL phenotype in Finns.

We found that in spouses, representing the control group, the MspI polymorphism in the promoter region of the apoA-I gene was associated with total cholesterol and apoB levels. The MspI polymorphism had earlier been associated with elevated HDL cholesterol, apoA-I, triglyceride, and apoB levels. We could also confirm the positive LD between MspI and SstI polymorphisms. The G→A nucleotide change that creates the MspI polymorphism has been reported to increase the transcription efficiency of apoA-I, probably by reducing the affinity of a regulatory factor.

We found the specific combination of haplotypes, which has previously been reported to be associated with FCHL status and plasma cholesterol and triglyceride values, in 11% of the probands and in 2% of their spouses, but the result was not statistically significant. None of the further analyses supported the association of any haplotype combination with the FCHL phenotype or lipid levels. There was no linkage between the studied markers and the FCHL phenotype. Because linkage analysis is dependent on the inheritance model used, several "model-free" statistical tests were performed, including nonparametric affected sib-pair, TDT, HRR, LD, logistic regression, and ANOVA. There was no excess allele sharing, allelic association, or LD between the studied polymorphisms and the FCHL phenotype. There was no statistically significant TDT or increased HRR detectable.

The fact that the studied polymorphisms showed no statistically significant association with the FCHL phenotype in Finnish FCHL families does not rule out that there could be other important polymorphisms in these genes that may be in LD with these polymorphisms in other populations. Other possible explanations for the difference between the results of the current study and earlier positive findings can probably be

### Table 5. The Frequencies of Haplotype Combinations in FCHL Family Members

<table>
<thead>
<tr>
<th>Halotype Combination</th>
<th>Hyperlipidemics (n=76)</th>
<th>Normolipidemics (n=287)</th>
<th>Spouses (n=114)</th>
<th>Probands* (n=27)</th>
<th>Predicted, %†</th>
<th>Significance, Pt</th>
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</thead>
<tbody>
<tr>
<td>111–111</td>
<td>0.283</td>
<td>0.396</td>
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<td>0.44</td>
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<td>61.6</td>
<td>1</td>
</tr>
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<td>1</td>
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<tr>
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<td>1</td>
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<td>0.004</td>
<td>0.000</td>
<td>0.037</td>
<td>62.4</td>
<td>0.10</td>
</tr>
</tbody>
</table>

*Probands were included in the hyperlipidemics group in logistic regression analysis.
†The percentage of individuals who were correctly assigned to hyperlipidemic or normolipidemic groups after including each haplotype combination in logistic regression analysis. (61.6% of the individuals were correctly assigned if their membership in a specific sibship was known.)
‡Significance of the likelihood-ratio test between the logistic regression models with and without the haplotype. The nonrandom distribution of frequencies of haplotype combinations between probands and spouses was tested by χ², and no significant deviations were noted.
found in different family selection, different criteria used for the determination of the FCHL affected status, different population gene frequencies, and partially the use of different statistical approaches.

In the study of Dallinga-Thie and coworkers,21 wherein the complex genetic contribution of the apoA-I/C-III/A-IV gene cluster to FCHL was presented, association studies were done with all normolipidemic and hyperlipidemic individuals in the FCHL families without adjusting for sibships. In that study, the individuals in the hyperlipidemic group were related and did not represent independent individuals, as is usually required in association analyses.22 In the hyperlipidemic relatives of that study, the rare alleles of \textit{XmnI} and \textit{MspI} polymorphisms were more frequent than in our study (a proper statistical comparison would require independent sampling). Also, the frequencies of haplotype combinations were clearly different between these 2 studies. The frequency of the most common haplotype combination, 1-1-1/1-1-1, was much lower in the Finnish FCHL family members than in the Dutch, and concurrently some haplotype combinations, ie, 1-1-1/1-1-2, 1-1-1/2-1-2, 1-1-2/1-1-2, 1-2-1/2-2-2, 2-1-1/2-2-2, and 2-2-1/2-2-2, were not encountered in the Dutch FCHL families. Two haplotype combinations, 2-1-2/2-1-2 and 1-2-1/1-2-1, were not seen in Finnish FCHL families. Those haplotypes in which the S2 allele combined with the X2 and M2 alleles were rare in Finnish and absent in Dutch FCHL families. The populations show minor differences in haplotype frequencies, but the frequencies of the haplotypes 2-2-2, 1-2-2, and 1-2-1 were also so low in the Finnish population that the different outcome with these rare haplotypes may have occurred by chance. We also used different patient selection (based on age- and sex-specific cutoff values) and different exclusion methods of familial hypercholesterolemia (based on age- and sex-specific cutoff values) and did not represent independent individuals, as is usually required in association analyses.22 In the hyperlipidemic relatives of that study, the rare alleles of \textit{XmnI} and \textit{MspI} polymorphisms were more frequent than in our study (a proper statistical comparison would require independent sampling).

Although we cannot exclude the possibility that the genes of the apoA-I/C-III/A-IV gene cluster act as a minor modifying factor in the pathogenesis of FCHL, we conclude that the current study does not support the direct involvement of these genes in FCHL in the Finnish FCHL families. The current study does not rule out the possibility that there could be functional mutations in other populations that are in LD or show other types of association with the studied polymorphisms.

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Appendix

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