Association of the FBXO11 Gene With Chronic Otitis Media With Effusion and Recurrent Otitis Media

The Minnesota COME/ROM Family Study

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Objective: The FBXO11 gene is the human homologue of the gene mutated in the novel deaf mouse mutant jeff (jf), a single gene model of otitis media. We have evaluated single nucleotide polymorphisms (SNPs) in the FBXO11 gene for association with chronic otitis media with effusion/recurrent otitis media (COME/ROM).

Design: A total of 13 SNPs were genotyped across the 98.7 kilobases of genomic DNA encompassing FBXO11. Data were analyzed for single SNP association using generalized estimating equations, and haplotypes were evaluated using Pedigree Disequilibrium Test methods.

Patients: The Minnesota COME/ROM Family Study, a group of 142 families (619 subjects) with multiple affected individuals with COME/ROM.

Main Outcome Measures: Genetic association of COME/ROM with polymorphisms in FBXO11.

Results: The FBXO11 SNPs are contained in a single linkage disequilibrium haplotype block. Ten of the 13 SNPs were sufficiently polymorphic in the sample to permit analysis. In univariate genetic analysis, 1 reference SNP (hereinafter rs) (rs2134056) showed nominal evidence of association to COME/ROM ($P = .02$), and 2 SNPs approached significance (rs2020911, $P = .06$; rs3136367, $P = .09$). In multivariable analyses, including known risk factors for COME/ROM (sex, exposure to smoking, attending day care centers, no prior breastfeeding, and having allergies), the evidence of independent association was reduced for each SNP (eg, rs2134056, from $P = .02$ to $P = .08$). In subsequent analyses using the Pedigree Disequilibrium Test, the association of FBXO11 SNP rs2134056 ($P = .06$) with COME/ROM was confirmed. Incorporating multiple SNPs in 2- and 3-locus SNP haplotypes, those haplotypes containing rs2134056 also exhibited evidence of association of FBXO11 and COME/ROM ($P$ values ranging from .03 to .10).

Conclusion: We have observed evidence consistent with an association between polymorphisms in FBXO11, the human homologue of the Jeff mouse model gene, and COME/ROM.

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other inflammatory disease. Animals with the jeff gene (hereinafter, Jeff animals) present with fluid and pus accumulation in the middle ear cavity, with diffuse mucosal inflammation, disruption of the eustachian epithelium, and reduction of the lumen owing to abnormal growths. The Jeff mutation appears to be fully penetrant and has been mapped to the distal region of mouse chromosome 17. A missense mutation has been identified in $Fbxo11$, a gene located on this region of mouse chromosome 17 (R. E. Hardisty-Hughes and S. D. M. Brown, unpublished data, 2006). The human ortholog, $FBXO11$, is located on chromosome 2 and encodes a 141-residue protein that belongs to the F-box family of genes. $FBXO11$ is characterized by the presence of a 50-amino acid sequence, the F-box, which functions in protein-protein interactions. The $FBXO11$ gene therefore represents a strong candidate gene for OM. This article examines polymorphisms in the $FBXO11$ gene for association in the human disease pathway for COME/ROM.

**METHODS**

**PARTICIPANTS**

All families and individuals in this study were recruited to participate in the genetic linkage study. Families who were excluded from the linkage study because they lacked an affected sibling pair with sufficient DNA were evaluated in this study. Study participants included families from previous studies of OM conducted by the Otitis Media Research Center at the University of Minnesota, Minneapolis, the members of the general public who responded to fliers posted around the university’s Academic Health Center, and otolaryngology clinic patients. Prospective families from previous studies were identified using OM history data collected in the original study on the participant (proband), parents, and siblings. Mothers from the families recruited from the general public and clinic patients were interviewed about the OM history of all family members to determine family eligibility. Data were collected at the study visit to determine phenotype (history of chronic OM or ROM), including findings from the otomicroscopic examination, multifrequency tympanometry, reported OM history, and medical record. An individual was considered to be affected if at least 2 of these sources had abnormal findings, or if 1 source showed abnormalities and middle ear or tympanometric findings were presumed to be definitive evidence of a history of COME/ROM (e.g., tympanosclerosis, atrophy, or tympanogram with high static admittance) were present. Subjects were examined by a neurootologist, and those with obvious craniofacial anomalies were excluded. Subjects were not examined by a dysmorphologist. Anatomic and morphologic traits and environmental factors in OM pathogenesis are highly interrelated, which made it difficult to impose specific criteria for inclusion or exclusion. Exclusion criteria included Down syndrome, cleft palate, or adopted siblings. A total of 142 families (619 individuals) participated in the candidate gene study. This includes 132 families who were included in the genome scan and linkage studies and 10 families who were recruited using the same ascertainment criteria and included only in this candidate gene study. DNA extracted from blood (94%) or buccal (6%) samples was used to assess candidate genes.

**GENOTYPING**

Thirteen single nucleotide polymorphisms (SNPs) in $FBXO11$, selected from the dbSNP public database, were genotyped in this study. We chose SNPs to cover the entire $FBXO11$ genomic region, including the 5' promoter region, exons, introns, and the 3'-untranslated region, and included rs2020911, rs3136367, rs3136371, rs3771285, rs3732191, rs330787, rs2651767, rs2134056, rs960106, rs2937345, rs874869, rs4992896, and rs7582252 (where rs indicates 1 reference SNP). Of these SNPs, rs7582252, rs2937345, and rs3136371 were not polymorphic and were not included in subsequent analyses.

We performed genotyping of the $FBXO11$ SNP polymorphisms by employing the MassARRAY SNP genotyping system (Sequenom Inc, San Diego, Calif) using a standard protocol. Total genomic DNA was purified from whole blood samples obtained from the subjects using PUREGENE DNA isolation kit (Gentra Inc, Minneapolis, Minn). We quantitated DNA using standardized fluorometric readings on a Hoeffe DyNA Quant 200 fluorometer (Hoefer Pharmacia Biotech Inc, San Francisco, Calif). Each sample was diluted to a final concentration of 5 ng/μL.

**STATISTICAL GENETIC ANALYSES**

We examined each pedigree for evidence of an incorrect family relationship by using genome scan data and PREST software. For each of the SNPs genotyped in this study, mendelian inconsistencies in their genotype assignment were examined using PEDCHECK software. Any genotypes inconsistent with mendelian inheritance (9 total [0.001%]) were converted to missing. Following pedigree and genotype correction, maximum-likelihood estimates of allele frequencies were computed using the largest set of unrelated individuals. All SNPs were then tested for departures from Hardy-Weinberg proportions. With multiple SNPs genotyped in the $FBXO11$ gene, the haplotype block structure and linkage disequilibrium (LD) between markers was assessed. Estimates of LD ($D^r$ and $r^2$) were computed also using the largest set of unrelated individuals and Dprime software. For families in which no founder was genotyped, the first affected offspring was used to estimate the statistics. Association between individual SNP and COME/ROM status was performed using a series of generalized estimating equation models. We adjusted for the correlation between subjects within a pedigree in the analyses by assuming exchangeable correlation among siblings within a pedigree and computing the sandwich estimator of the variance. The sandwich estimator is also denoted the robust or empirical estimator of the variance and is robust to misspecification of the correlation matrix because it estimates the within-pedigree correlation matrix from the first and second moments of the data. Broadly speaking, this method is comparable to a logistic regression analysis with adjustment for familial correlation among family members. As such, the unaffected members of the pedigrees provide allele counts that correspond to control genotype frequencies. For each SNP, the 2-df overall test of genotypic association was performed, and, if the overall genotypic association was significant, 3 individual contrasts defined by the a priori genetic models (dominant, additive, and recessive) were computed. If the overall genotypic association was not significant, the a priori contrasts were examined after adjusting for the 3 comparisons using a Bonferroni adjustment, consistent with the Fisher protected least significant difference multiple-comparison procedure. Tests of association between SNP and COME/ROM were computed adjusting for sex, number of smokers in the household, prior breastfeeding, presence of allergies, and past day care center attendance.

Haplotypes associations between the COME/ROM and the $FBXO11$ SNPs were also assessed. Haplotypes were constructed and the analyses of the 2- and 3-marker haplotypes were completed using a generalized estimating equation analy-
sis as described herein, except the quasi-likelihood was weighted by the probability for each possible haplotype-genotype for an individual. Each individual was entered in the generalized estimating equation analysis once for each haplotype-genotype possibility, weighted by the haplotype-genotype probability. Thus, the weight for each individual sums to 1. The weighted generalized estimating equation analyses were completed as described herein, using the sandwich estimator of the variance to account for the within-cluster correlation.

To test for allelic association while accounting for potential population stratification, the Pedigree Disequilibrium Test (PDT) was employed.27 The PDT was performed using both single SNPs as well as 2- and 3-marker haplotypes within FBXO11. The PDT operates on the same principle as the transmission disequilibrium test: alleles transmitted to cases create genotype frequencies for controls, whereas alleles that are not transmitted comprise allele frequencies for controls. The PDT is more powerful than the transmission disequilibrium test, and it allows analysis of transmission of alleles with all available family data. The PDT is a valid test of association even in the presence of population substructure and it maintains the analysis of transmission within families.27

**RESULTS**

**PARTICIPANTS**

A total of 142 families (619 individuals) participated in the candidate gene study. Of the participants in this study, 40% were classified as affected (2 or more sources of data positive for COME/ROM, or definitive examination findings), and 60% were unaffected. Most were white (94%) and non-Hispanic (99%). Risk factors were prevalent, most participants had attended day care centers, about 20% reported allergies, and exposure to smokers was common (Table 1).

**Table 1. Descriptive Data for the Minnesota COME/ROM Family Study**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pedigrees, No.</td>
<td>142</td>
</tr>
<tr>
<td>Total subjects, No. (% female)</td>
<td>619 (53)</td>
</tr>
<tr>
<td>Smokers in the home, mean ± SD (median)</td>
<td>0.75 ± 0.85 (0.0) [618] [No. of subjects]</td>
</tr>
<tr>
<td>Exposed to smoking in household, No. (%)</td>
<td>618 (49)</td>
</tr>
<tr>
<td>Attending day care centers, No. (%)</td>
<td>617 (52)</td>
</tr>
<tr>
<td>Prior breastfeeding, No. (%)</td>
<td>591 (57)</td>
</tr>
<tr>
<td>Allergies, No. (%)</td>
<td>618 (22)</td>
</tr>
</tbody>
</table>

Abbreviation: COME/ROM, chronic otitis media with effusion/recurrent otitis media.

**Figure.** Genomic map of FBXO11. The gray boxes represent coding exons, and the ruler at the bottom shows the relative location and spacing of genotyped single nucleotide polymorphisms in kilobases (kb). FBXO11 and the neighboring MSH6 (homologue of MutS 6) genes are oriented in a tail-to-tail fashion.

The FBXO11 gene consists of 23 exons spanning more than 98 kilobases (kb) of genomic sequence arranged in a tail-to-tail configuration with the MSH6 guanine/thymine mismatch repair gene, resulting in an overlapping sequence of 32 base pairs in their 3’ untranslated regions (Figure).28 Thirteen SNPs were genotyped across the 98.7 kb of genomic DNA encompassing the FBXO11 gene in 619 individuals from 142 families in the Minnesota COME/ROM Family Study. The Figure also shows the location and distribution of the SNPs relative to the genomic structure of the gene. FBXO11 is a complex gene, and the 13 SNPs were chosen to systematically cover the genomic region with a density of 1 SNP per 7.6 kb. A large gap of 31.5 kb exists between exons 2 to 15 in which no SNPs were identified that were polymorphic in the study subjects. The current set of 13 SNPs is, however, contained in a single LD haplotype block. Only 1 of the SNPs is in an exon (rs3136371), but this SNP is in the non-coding 3’ untranslated region. Bioinformatic analysis did not identify SNPs in other exons. After genotyping, all SNPs were evaluated for Hardy-Weinberg equilibrium and pairwise LD. The SNPs with minor allele frequencies greater than 0.10 exhibited genotypic frequencies that were consistent with Hardy-Weinberg proportions. D’ values were greater than 0.95 for all SNPs with minor allele frequencies greater than 0.10, suggesting that the entire FBXO11 gene is encompassed by a single LD haplotype block (data not shown).

Single SNP association analysis was performed using general estimating equation methods. Ten of the 13 SNPs were sufficiently polymorphic in the sample (ie, they had minor allele frequencies >0.10) to permit analysis. In analysis of the COME/ROM phenotype summarized in Table 2, SNP rs2134056 (located in the distal portion of intron 1; Figure) exhibited nominal evidence of asso-
fbx1 proteins...f-box...FBXO11 protein is unknown, a missense mutation in the mouse Fbxo11 gene (R. E. Hardisty-Hughes and S. D. M. Brown, unpublished data, 2006) in the jeff mouse model of chronic proliferative OM makes human FBXO11 a candidate for involvement in COME/ROM. Evidence for mutation of Fbxo11 leading to OM in the jeff mouse model illustrates the power of modern genetic methods to identify novel pathways that, based on biological understanding alone, might not be implicated in the pathophysiologic traits of a disorder such as COME/ROM.

**PDT ANALYSIS**

In addition to single SNP associations, there is the possibility that a combination of SNPs (ie, a haplotype) confers increased risk for COME/ROM. We performed haplotype association analysis with the SNP data using the PDT. Table 3 shows the results of analyses with *P* = .10. In single SNP analysis with a PDT, the rs2134056 SNP showed some evidence of association with COME/ROM (*P* = .09). Haplotype analysis was performed using a sliding window approach, so that combinations of 2- and 3-adjacent SNPs were tested sequentially. Consistent with the single SNP analysis, the 2- and 3-adjacent SNP combinations that include rs2134056 (eg, rs2651767 and rs2134056; *P* = .03) reveal evidence for association with COME/ROM.

FBXO11 is a member of the FBXO subfamily of proteins that have an F-box but no recognized substrate-binding region. Although the specific function of the FBXO11 protein is unknown, a missense mutation in the mouse Fbxo11 gene (R. E. Hardisty-Hughes and S. D. M. Brown, unpublished data, 2006) in the jeff mouse model of chronic proliferative OM makes human FBXO11 a candidate for involvement in COME/ROM. Evidence for mutation of Fbxo11 leading to OM in the jeff mouse model illustrates the power of modern genetic methods to identify novel pathways that, based on biological understanding alone, might not be implicated in the pathophysiologic traits of a disorder such as COME/ROM.

**COMMENT**

Some comment as to the relevance of the jeff mouse model to COME/ROM seems appropriate. For example, susceptibility to OM in both the jeff mouse and the study subjects could be due to subtle craniofacial abnormalities leading to eustachian tube dysfunction. Hearing loss in the jeff mouse was of a mixed nature. The raised thresholds obtained for the cochlear nerve response were beyond what would be expected for a purely conductive loss. Also, some jeff mice showed an abnormally low endocochlear potential indicating involvement of the stria vascularis in the hearing loss. Humans with middle ear disease often show sensorineural components to their hearing loss, potentially as a consequence of damage to the lateral wall of the cochlea. Also, in other animal models, cochlear disease has been seen in experimentally induced OM, which is consistent with the raised endocochlear potentials seen in jeff mice.

The craniofacial abnormality in jeff mice was evident on the C3H/HeH background (the laboratory strain on
which the mutant was originally maintained, and the background used in previous studies. When on a different genetic background (C57BL/6), the penetrance of the abnormal face phenotype diminishes and is not always coincident with the presence of OM (R. E. Hardisty-Hughes and S. D. M. Brown, unpublished data, 2006). For these reasons, OM in the jff mouse model does not seem to be solely due to a structural abnormality that results in dysfunction of the eustachian tube.

We have performed a systematic evaluation of FBXO11 in a well-characterized population of COME/ROM families from the COME/ROM Family Study. Multiple polymorphisms have been genotyped in an effort to provide a comprehensive survey of gene variation in the gene. The SNP genotyping data are consistent with the FBXO11 being contained in a single linkage disequilibrium haplotype block. Univariate, multivariate, and haplotype analysis using a variety of analytical approaches provide evidence consistent with the genetic involvement of human FBXO11 in COME/ROM. We note that the magnitude of the significance for association is not dramatic, which is consistent with the observation that the FBXO11 region was not detected in our previous genome scan for linkage. However, with a strong a priori hypothesis, these results suggest additional analyses of FBXO11 in other relevant populations.

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REFERENCES