

Genetic Contributors to Otitis Media: Agnostic Discovery Approaches

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Abstract Otitis media (OM) is the most common disease in children in the United States, with about \$5 billion spent each year in direct and indirect costs. OM is the number one reason for pediatric antibiotic usage and surgery, although treatment options are limited. Numerous studies have established the high heritability of OM and a genetic contribution to OM pathogenesis. Candidate gene studies have highlighted the roles of inflammation, mucin secretion, and pathogen recognition, but this approach is unable to identify novel pathways to target for treatment or screening purposes. Here, we review the current literature on agnostic approaches to discover novel genes and pathways involved in OM pathogenesis.

Keywords Otitis media · Discovery · Genome · Candidate genes · Population studies · Genetics

Introduction

Each year in the United States, over \$5 billion is spent on healthcare for inflammation of the middle ear (ME) known as otitis media (OM) in children [1, 2]. Almost all children are

estimated to have at least one episode of acute otitis media (AOM) by age 3 [3]. Some children who have AOM develop subsequent infections, resulting in recurrent otitis media (ROM) and/or chronic otitis media with effusion (COME) [4]. COME/ROM is a multifactorial disease arising from the interactions between viral upper respiratory infection, bacterial colonization, host genetic factors, and environmental risk factors.

There is considerable evidence for a genetic contribution to COME/ROM. Twin, sibling, and family studies show high heritability for OM, with heritability referring to the proportion of the phenotypic variance due to inherited factors. Estimated heritability of OM in a Norwegian study of 2,750 twin pairs was 0.45 for males and 0.74 for females [5]. Another study completed at the University of Pittsburgh followed 175 same-sex twin or triplet sets and concluded that the heritability of the mean time with otitis media with effusion (OME) in the first 2 years of life was 0.73, with a slightly lower heritability of time with OME found in boys [6]. In a U.K. study of 715 monozygotic twins and 658 dizygotic twins, heritability of acute OM was 0.57, and heritability of chronic airway obstruction, a strong predictor of COME, was 0.72 [7]. A study of the heritability of COME/ROM in Helsinki, Finland, using a cohort of 1,279 children and their parents estimated heritability to be 0.385 for ROM, 0.221 for COME, and 0.478 for any OM severity [8]. The strong genetic component of OM observed in these studies provides a strong rationale for genome-scale studies.

Many studies have focused on determining the role of specific candidate genes in the pathogenesis of OM and increased susceptibility to OM, extensively reviewed previously [9]. The majority of genes investigated in these studies are involved in inflammation and the immune response, since those are biologically plausible and/or identified in mouse studies.

A recent candidate SNP study was completed in 100 OM cases and 100 controls using 192 tag-SNPs in candidate genes [10]. No SNPs reached significance when adjusted for multiple testing due to lack of power to detect association, though SNPs from *TLR4*, *MUC5B*, *SMAD2*, *SMAD4* require follow

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up in larger, independent populations as they had P values <0.05 before adjusting. Agnostic approaches to gene discovery provide a means of identifying new genes and pathways underlying susceptibility to OM by probing evidence for association in a comprehensive scan of the whole genome. To date, only two linkage studies and two genome-wide association studies (GWAS) of OM have been completed.

Despite the fact that COME is a complex, multifactorial disease, several genetic loci have successfully been discovered. Agnostic, genome-scale studies to date comprise two linkage scans and two GWAS. Two follow-up studies of the linkage results include a fine mapping approach and a replication study. The designs and results of these studies are summarized in Table 1.

Linkage Studies

Linkage studies employ genetic analysis in the context of a family-based design including sibling pairs, trios, larger sibships, nuclear families, or extended pedigrees. Linkage analysis leverages knowledge about the relationship status between individuals in the same family (e.g., siblings, parent–offspring, uncle–nephew) to infer the expected distribution of alleles shared identical by descent. A basic assumption underlying linkage analysis is that, for genomic regions playing a role in the disease of interest, affected individuals from the same family will inherit more chromosomal segments (or alleles) in common than expected by chance. Likewise, unaffected individuals would be expected not to share these same alleles with the affected individuals. In this way, it is possible to identify genomic regions that show evidence of linkage with the disease of interest.

Linkage studies are considered robust among geneticists because the family design protects the study from environmental noise and population stratification. In general, linkage studies do not provide fine resolution of the identified linkage peaks because they rely on genetic recombination events within families to narrow down the genomic region of interest. Recent studies have demonstrated that large linkage regions may represent multiple rare (private) variants responsible for disease inheritance within different families [11, 12].

University of Minnesota Genome Wide Linkage Scan

The University of Minnesota (UMN) linkage scan was the first study to investigate genetic determinants of COME/ROM across the genome [13]. This study recruited COME/ROM affected children along with siblings and parents, with a total of 692 individuals from 153 families. The Center for Inherited Disease Research (CIDR) genotyped 404 microsatellite markers in the COME/ROM families. After removal of genotypes and samples in quality control analyses, a total of 248,219 genotypes were generated in 591 samples, 238 of

which were affected. Significant evidence of linkage was found on 10q (LOD 3.78; $P=3.0\times 10^{-5}$) and suggestive evidence of linkage was found on 19q (LOD 2.61; $P=5.3\times 10^{-4}$). Multipoint NPL analyses also provided evidence of linkage to COME/ROM on chromosome 19q (LOD 2.53). Analyses conditional for linkage at chromosomes 10q and 19q resulted in increased support for linkage at 3p [unconditional LOD 0.60; conditional (10q) LOD 2.43; conditional (19q) LOD 1.84]. The chromosome 19q linkage region was a large region encompassing 90 genes with candidate genes with known roles in inflammation and BMP and TGF β signaling.

University of Minnesota Fine Mapping of Chromosome 19q

The goal of this study was to fine map the chromosome 19q region previously found in the UMN genome scan of COME/ROM to further localize the linkage signal [14]. In this study, a total of 607 individuals from 139 families were used in analyses which included all from the UMN genome scan with the addition of six new families recruited with the same criteria. To fine map the chromosome 19q region, there were 1,536 SNPs chosen for genotyping, 1,492 of which were non-synonymous and synonymous coding SNPs and 44 of which were Ancestry Informative Markers (AIMs) from across the genome. Genotyping using Illumina's GoldenGate assay was carried out at CIDR. After removal of SNPs during quality control analyses, there were 1,091 SNPs available for analyses. Significant evidence for linkage between COME/ROM and chromosome 19q was found at 11.5 cM (LOD 3.75; $P=1.6\times 10^{-5}$) which is 63.4 Mb physical distance (hg18) when marker-to-marker LD was modeled at $r^2>0.5$ or $r^2>0.2$. Due to the chromosome 19q locus, the recurrence risk of COME/ROM in a sibling of an affected individual is twice as high as that in the population. The LOD-1 Score region of this locus (61.6–63.8 Mb, hg18) contains 93 known and hypothetical genes. Within this group, there are many potential COME/ROM susceptibility gene candidates including *ZNF8*, binds with *SMAD1* to repress of BMP and TGF β signaling during development in mice [15]; *NLRP13*, *NLRP5*, and *NLRP8*, members of the inflammasome complex which is an important regulator of the innate immune response [16–18]; and Alpha-1-B glycoprotein (*A1BG*) associated with severe inflammation [19].

University of Pittsburgh Genome Wide Linkage Scan

The University of Pittsburgh (UPitt) linkage scan also sought to discover linkage between novel loci and otitis media susceptibility [20]. This study sample included at least two full siblings who had history of tympanostomy tube surgery due to a significant history of OM, along with their parents and other full siblings with no history of tube surgery. Genotyping was carried out using two versions of the Affymetrix's GeneChip®

Table 1 Genome-wide studies of otitis media

Study	University of Pittsburgh ^{20, 22}	Western Australia Family Study of Otitis Media (WAFSOM) ²¹	Western Australia Pregnancy Cohort (Raine) Study ²¹	University of Minnesota ^{13, 14, 22}
Phenotype definition	- Affected if he/she had tympanostomy tube surgery at least once for recurrent/persistent OM - Unaffected if he/she had never had tympanostomy tube surgery and had no known history of recurrent/persistent OM - Required one visit to the ENT research Center at the Children's Hospital of Pittsburgh - Detailed history regarding recurrent/persistent OM and risk factors was collected for each family member - Medical records were obtained when possible - Tympanograms were obtained	- Probands were recruited if they had tympanostomy tube surgery for RAO/COME prior to 3 years of age. - Parents and siblings were recruited. - Affected siblings were considered as affected if defined as having ≥ 3 episodes of AOM by 3 years of age and/or COME with tubes inserted or recommended. - Parents completed a study-specific questionnaire. Information regarding risk factors was also collected. - Family members with no history of RAO/COME were classified as unaffected.	- Affected status was assessed using questionnaires and clinical examinations at ages 1, 2, and 3. - Cases defined by examining clinical records from the first 3 years of life for record of the presence of: - Inflamed, retracted, or scarred tympanic membrane - Middle ear effusion - Tympanostomy tubes - Parental reports were also used to define cases if they had ≥ 3 episodes of AOM by 3 years of age. - Controls were children with no evidence of AOM by 3 years of age.	- Affected if he/she had tympanostomy tube surgery for COME/ROM or if there were at least 2 positive findings from the following data sources: - Ear examination to determine presence of OM sequelae without ENT knowing the subject's prior OM history - Tympanometric testing was performed in subjects at three frequencies to detect abnormal middle ear mechanics, and hearing was screened at 20 dB for speech frequencies - Questionnaire/interview about OM history, risk factors, and related conditions - Medical record review
Population	≥ 2 full siblings with history of tubes due to a significant history of OM - Parents and other full siblings with no history of tubes - Exclusion criteria: major congenital malformations, medical conditions with a predisposition for OM, cared for in the Intensive Care Unit as a neonate, been assisted on ventilation, or having known sensorineural hearing loss.	- Family-based cohort with probands, parents, and any affected siblings recruited. - Exclusion criteria: predisposing medical conditions including immune deficiencies, craniofacial abnormalities, and specific genetic syndromes.	- Longitudinal cohort of children whose mothers were recruited from antenatal clinics. - Exclusion criteria: diagnosed with cleft palate	- Probands (children treated with tympanostomy tubes for COME/ROM) and their families (parents, siblings, and other relatives) were recruited - Exclusion criteria: family member who is adopted or if diagnosed with Down Syndrome, craniofacial anomaly, another genetic disorder with otologic complications, or tympanostomy tubes for a diagnosis other than OM
Results	- Evidence of linkage at 17q12–LOD 2.83 - Suggestive linkage at: 10q22.3 7q33 6p25.1 4p15.2 - Combined Caucasian and African American dataset improved linkage at 10q22.3 from $P=1.81 \times 10^{-3}$ to $P=2.6 \times 10^{-4}$ - Replication study of UMN top GWAS hits found significant association at rs10497394 ($P=4.7 \times 10^{-5}$)	Investigated replication of Raine cohort GWAS loci but did not find significant association.	- Top associations: - rs13386850 ($P=1.32 \times 10^{-6}$) - rs13408922 ($P=1.32 \times 10^{-6}$) - Top hit from PC adjusted analysis of full imputed dataset: - rs6755194 ($P=8.3 \times 10^{-7}$) - New candidate genes for OM - CAPN14 - GALNT14	- Evidence of linkage at 10q–LOD 3.78, $P=3.0 \times 10^{-5}$ - Evidence of linkage at 19q–LOD 2.61, $P=5.3 \times 10^{-4}$ - Conditional linkage at 3p: [10q] LOD 2.43 [19q] LOD 1.84 - Most significant association in discovery GWAS=rs1110060 ($P=9.1 \times 10^{-7}$) - Most significant result=rs10497394 - GWAS $P=2.9 \times 10^{-5}$ - Replication $P=4.7 \times 10^{-5}$ - Meta-analysis $P=1.52 \times 10^{-8}$ - New candidate genes for OM - KIF7 - TICRR

Human Mapping 10 K Array in a total of 1,506 individuals, including 1,431 European American and 75 African American individuals. After removal of SNPs for quality control measures, 8,802 SNPs were tested for linkage to recurrent/persistent OM. Analyses of European American families only detected the most significant region of linkage at 17q12 (LOD 2.83; $P=7\times 10^{-5}$) and four other peaks with suggestive P values: 10q22.3 (1.81×10^{-3}), 7q33 ($P=1.05\times 10^{-3}$), 6p25.1 (LOD 2.25; $P=2.61\times 10^{-3}$), and 4p15.2 ($P=3.01\times 10^{-3}$). Analyses using the combined European American and African American families strengthened evidence of linkage to recurrent/persistent OM at 10q22.3 ($P=2.6\times 10^{-4}$) but significance at the other peaks decreased. In the chromosome 17q12 linkage peak, there are OM susceptibility candidate genes including adaptor-related protein complex 2, beta 1 subunit (*AP2B1*), a gene associated with the down-regulation of CD8 and a cluster of chemokine C-C motif ligand (*CCL*) genes including *CCL5* which has previously been associated with OM. This study did not confirm the regions found to be significantly linked to COME/ROM in the UMN linkage study, 10q26.3 and 19q13.43. The findings of this study need further fine mapping, replication, and functional studies to determine causal SNPs increasing risk of recurrent/persistent OM.

Genome-Wide Association Studies

While linkage studies examine patterns of inheritance within families, genome-wide association studies (GWAS) use a population-based approach to examine evidence of allelic association with disease outcomes. Following genome-wide genotyping, each genetic variant or SNP is examined one at a time for evidence of association with disease susceptibility. GWAS draw across entire populations such that the resulting association signals are narrowed to the linkage disequilibrium (LD) blocks surrounding the causal variant(s) of a genetic locus. In this manner, GWAS typically narrow the genetic locus of interest to only one or a dozen SNPs, providing comparatively finer resolution than linkage studies. As GWAS are performed at the population level, they face the limitations of large-scale epidemiologic studies, including issues of population stratification and confounding. Genetic associations may be reported for common variants. Rare and infrequent variants cannot typically be identified through GWAS, as such variants are typically underpowered in population-based samples. Furthermore, the ability to detect association for a particular genetic locus depends on SNP density, coverage, and patterns of LD within the genetic region harboring the causal variant(s) for the disease of interest.

Western Australia Pregnancy (Raine) Cohort GWAS of Acute/Chronic OM

This was the first study to investigate genetic contributors to OM using a genome-wide associations approach [21]. Analyses included all subjects with available GWAS data from the Western Australia Pregnancy (Raine) Cohort. Genotype data was generated on the Illumina 660 W Quad Beadchip at the Centre for Applied Genomics. There were 2,524,817 SNPs (genotyped and imputed) available for analysis in 416 cases and 1,075 controls. No SNP exceeded genome-wide significance ($P<5\times 10^{-8}$). There were 46 variants that, when adjusted for 2 principal components (PCs) to account for population stratification, showed associations at $P_{\text{adj-PCA}}<10^{-5}$. The top associations in this study were found in the region upstream of Calpain 14 (*CAPN14*) on chromosome 2, and this candidate gene was confirmed when the full imputed dataset was analyzed and the top association was rs6755194 ($P_{\text{adj-PCA}}=8.3\times 10^{-7}$) which lies in an intron of *CAPN14*. Novel candidate genes determined based on the location of the SNPs with the most significant association with OM included *CAPN14* and *GALNT14* and those determined based on a gene-based analysis included *BPIFA3* and *BPIFA1*. These novel candidate genes may have putative functional roles in susceptibility for OM. Additionally, genes that are a part of or interact with the TGF β pathway were highlighted in their pathway analysis. The most significant associations were investigated for replication in WAFSOM, an independent OM population, but none of the associations were replicated. Lack of replication is postulated to be due to phenotypic differences in the populations and sample size. In conclusion, though no SNP exceeded genome-wide significance, we now have novel candidate genes found in the TGF β pathway which has previously been associated with OM.

University of Minnesota GWAS of COME/ROM

Using the UMN family population, a GWAS was conducted to discover novel variants associated with COME/ROM [22]. In this study, there were 602 individuals from 143 families genotyped at 324,748 SNPs using the Illumina Human CNV370-Du0 DNA BeadChip. The strongest association with COME/ROM in this study was rs1110060 ($P=9.1\times 10^{-7}$) which is located in the intron of kinesin family member 7 (*KIF7*). No findings were robust to Bonferroni correction ($P=0.05/324,748=1.54\times 10^{-7}$). A subsequent replication study of 53 SNPs from the discovery GWAS using the University of Pittsburgh family population of otitis media found significant association at rs10497394 ($P=4.7\times 10^{-5}$), a SNP in a large intergenic region on chromosome 2 [22]. Meta-analysis confirmed replication at this SNP ($P=1.52\times 10^{-8}$) and showed evidence of association at chromosome 15 SNPs rs1110060 ($P=4.09\times 10^{-5}$) and rs10775247 ($P=3.00\times 10^{-4}$).

The chromosome 2 SNP lies within a large intergenic region with many genomic and functional elements including transcription factor binding sites, a pseudogene, DNaseI hypersensitive regions, and a large active enhancer region (H3K27Ac and H3K4me1 marks). The chromosome 15 SNPs lie in adjacent genes *TICRR* and *KIF7*, neither of which has yet been associated with higher susceptibility of COME/ROM. In conclusion, this study identified and replicated a novel SNP on chromosome 2 to be associated with COME/ROM and identified two genes on chromosome 15 as candidate genes of OM which will require further study.

Conclusions

Although candidate gene and animal model approaches have highlighted important pathways in the pathogenesis of OM, additional genetic contributors remain to be identified to explain the heritability of this disorder. Genome-scale agnostic approaches have the advantage that they do not presume prior knowledge of disease pathogenesis, and thus hold the promise of novel insights into mechanism. Unfortunately, in the field of OM research, there have been insufficient genomic scans and even fewer follow-up investigations of these results. Promisingly, the association at rs10497394 discovered in the UMN GWAS was replicated in the University of Pittsburgh family population, an independent population of OM.

Results of these genome-wide studies have demonstrated evidence for mechanisms of OM pathogenesis that have not yet been discovered using candidate gene approaches. These novel mechanisms could provide new screening and treatment options which could reduce recurrent and chronic OM occurrences. The two GWA studies both discovered novel candidates, including novel candidates for OM include *CAPN14* and *GALNT14*, and novel candidates for COME/ROM include *KIF7* and *TICRR*. Further investigation of these hits could uncover novel mechanisms of OM pathogenesis. These functional assays will be used to assess the role of the associated SNPs in alternative splicing of *KIF7*, validate a SNP as an exon-QTL in tissues relevant to OM, and evaluate the role of associated SNPs on enhancer activity. On top of discovering novel candidates, these studies have demonstrated the importance of the TGF β pathway, originally discovered using ENU mutagenesis screens, in OM.

Many linkage studies still have not been narrowed down to a single gene or gene cluster and GWAS results have yet to confirm the identity of the underlying causal variant. SNPs are genotyped in these studies are selected on the basis of genomic coverage and patterns of LD. The SNP associated with the trait of interest could be in high or even complete LD with other SNPs inherited from a common ancestor and therefore cannot be distinguished from each other by association methods. One way to hone in on the causal variants is to

examine more diverse populations which have different patterns of LD and can narrow down regions to be functionally examined. The 1000 Genomes Project [23] has generated WGS data from multiple populations including those from European, East Asian, West African, and South Asian ancestry and populations from the Americas as a publicly available resource to the genomics community. The HapMap project [24] characterizes the patterns of LD in a subset of these populations. These resources, along with recruitment of diverse populations with OM, can help us narrow down linkage and GWAS signals.

Lack of replication between genetic studies of OM has been attributed to phenotypic heterogeneity due to inconsistent application of clinical criteria and lack of power due to sample size. There is a critical need for more studies of larger sample size and more diverse populations to discover novel mechanisms of OM. Additional independent studies will enable the investigation of replication of candidate genes and suspected loci from genomic studies. In order to avoid lack of replication due to heterogeneous definitions of OM and its various severities, there is a need for consistency in phenotype definitions and recruitment criteria spanning both the clinic and research studies. The International Consortium of the Genetics of OM (OTIGEN; <http://www.otigen.org/>) was initiated by otitis media investigators with the common intention to resolve the genetic contributions to OM. In order to carry out the goal of OTIGEN, the investigators of OTIGEN strive to bring consistency to OM phenotypic definitions and strategize larger studies and collaborations to replicated findings of individual studies.

In summary, these genome-wide studies provide new insights to otitis media research which may provide the necessary loci for the development of novel screening or treatment tools. Candidate gene studies are able to verify biological knowledge, but agnostic approaches are not limited and are able to discover new targets. Efforts need to be placed on consistency in diagnosis, recruitment, and phenotyping to allow for more efficient population studies.

Compliance with Ethics Guidelines

Conflict of Interest Ani Manichaikul has received grant support from the Hearing Health Foundation.

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Human and Animal Rights and Informed Consent This article does not contain any studies with animal subjects performed by any of the authors. With regard to the authors' research cited in this paper, all procedures were followed in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 2000 and 2008.

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