Genetic and Environmental Determinants of Otitis Media in an Indigenous Filipino Population

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Abstract

Objective. To identify genetic and environmental risk factors for otitis media in an indigenous Filipino population.

Study Design. Cross-sectional study.

Setting. Indigenous Filipino community.

Subjects and Methods. Clinical history and information on breastfeeding, tobacco smoke exposure, and swimming were obtained from community members. Heads of households were interviewed for family history and personal beliefs on ear health. Height and weight were measured. Otoscopic findings were described for the presence and character of perforation or discharge. An A2ML1 duplication variant that confers otitis media susceptibility was Sanger sequenced in all DNA samples. Co-occurrence of middle ear bacteria detected by 16S rRNA gene sequencing was determined according to A2ML1 genotype and social cluster.

Results. The indigenous Filipino population has a ~50% prevalence of otitis media. Young age was associated with otitis media (4 age strata; P = .004); however, age was nonsignificant as a bistratal or continuous variable. There was no association between otitis media and sex, body mass index, breastfeeding, tobacco exposure, or deep swimming. In multivariate analyses, A2ML1 genotype is the strongest predictor of otitis media, with an odds ratio of 3.7 (95% confidence interval: 1.3-10.8; P = .005). When otitis media diagnoses were plotted across ages, otitis media was observed within the first year of life, and chronic otitis media persisted up to adulthood, particularly in A2ML1-variant carriers.

Conclusion. Among indigenous Filipinos, A2ML1 genotype is the primary risk factor for otitis media and the main determinant of disease progression, although age, the middle ear microbiome, and social clusters might modulate the effect of the A2ML1 genotype.

Keywords

A2ML1, indigenous population, microbiome, middle ear, otitis media, Philippines

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Otitis media is the top reason for clinical consult and antibiotic use in US children, with an annual cost >$5 billion.\textsuperscript{1-3} Worldwide, the incidence and prevalence of otitis media are highest in sub-Saharan Africa and Asia and in indigenous populations, which bear the burden of health care use and hearing loss mostly due to otitis media.\textsuperscript{4-6} Due to the high prevalence of chronic suppurative otitis media and its complications in developing countries, it was recently proposed that chronic otitis media be classified as a neglected tropical disease.\textsuperscript{7} Despite adequate vaccine coverage, otitis media prevalence can still be high in indigenous populations.\textsuperscript{8} To improve prevention and treatment regimens, better knowledge of otitis media pathophysiology is required, particularly in those with increased susceptibility to otitis media due to risk factors such as young age, crowded household, day care attendance, ethnic differences, and human mutation.\textsuperscript{9,10}

An indigenous community in the Philippines was identified to have a very high prevalence of otitis media (Figure 1). Within this highly intermarried community, a large pedigree was used to identify a genetic variant: a duplication c.2478_2485dupGGCTAAAT, p.(Ser829Trpfs*9), within the A2ML1 gene that induces susceptibility to otitis media.\textsuperscript{10} A2ML1 encodes α-2 macroglobulin-like-1 protein, or A2ML1, which is localized to middle ear mucosal epithelium and is similar in sequence to the protease inhibitor α-2 macroglobulin, or A2M.\textsuperscript{10} It is predicted that A2ML1 and A2M have similar structures and perform overlapping protective functions within the middle ear (eg, trapping bacterial or host inflammatory proteases) such that dysfunctional A2ML1 can result in mucosal damage.\textsuperscript{10} Audimetric testing in a few individuals from this population documented hearing loss due to otitis media.\textsuperscript{11} Microbiome studies on middle ear swabs from selected persons with chronic otitis media revealed unique bacterial profiles according to carriage of the A2ML1 variant.\textsuperscript{12} In this report, environmental variables, carriage of the A2ML1 variant, and middle ear bacteria were analyzed as risk factors for otitis media within the indigenous Filipino population. Our findings suggest that the A2ML1 genotype is the strongest predictor of otitis media occurrence within this population, with some evidence of modulation by age and social clusters in terms of disease onset, progression, and carriage of specific bacteria within the middle ear.

**Methods**

An indigenous island community within the central region of the Philippines was reported by community members to have a high prevalence of otitis media. The indigenous (Negrito) Filipinos are the original inhabitants of the island community but have suffered racial segregation over centuries due to their dark-colored skin, curly hair, flat noses, and short stature. These physical features of Negritos are not disease related, and no other infectious, craniofacial, skeletal, cardiopulmonary, mental, genetic, or immune diseases co-occur with nonsyndromic otitis media or are as prevalent within the community. Racial segregation has fostered intermarriage, and the majority of community members can be traced back to a few founders over 6 generations. The community was visited 5 times over 8 years, with a relatively stable population of 200. Ethical approval for conduct of the study was granted by the National Commission on Indigenous Peoples, the Research Ethics Board of the University of the Philippines Manila, and the Institutional Review Board of the Baylor College of Medicine and Affiliated Hospitals. In addition to community consent through the National Commission on Indigenous Peoples, informed consent was obtained from study participants.

For each person who provided saliva using the Oragene DNA saliva kit (DNA Genotek, Ottawa, Canada), DNA samples were isolated and genotyped for the A2ML1 variant via Sanger sequencing.\textsuperscript{10} For 16 people with chronic otitis media, carriage of specific bacterial taxa within the middle ear was determined through 16S rRNA gene profiling, as previously described.\textsuperscript{12} In brief, middle ear swabs were collected by rubbing sterile short polyester-tipped Pur-Wrap swabs (Puritan Medical, Guilford, Maine) against middle ear mucosa and the edges of eardrum perforations and by soaking the swab on discharge when present. The swab stem was cut with sterile scissors and placed into a tube, which was closed, sealed, and labeled. All samples were stored in a −20°C freezer until shipped on dry ice to the Baylor College of Medicine Alkek Center for Metagenomics and Microbiome Research. From each swab specimen, DNA was isolated with the PowerSoil DNA kit (MO BIO Laboratories, Carlsbad, California) and submitted for 16S rRNA gene sequencing. The 16S rRNA gene V4 region was amplified by polymerase chain reaction and sequenced in an Illumina MiSeq via the 2 × 250-base pair paired-end protocol. Analysis was performed with an in-house pipeline that includes custom analytic packages that provide summary statistics and quality control measurements for validating built-in controls and characterizing microbial biodiversity.
communities across large numbers of samples or groups. In this report, only the most significant, abundant, or notable bacterial genera were selected for study.12 Participating subjects were interviewed for clinical history and personal information on other risk factors for otitis media, such as breastfeeding in infancy, exposure to tobacco smoke, and swimming in deep water (ie, ≥3 m). To determine environmental factors within the community, a questionnaire was administered to the heads of households, including queries on household income, highest level of education within the household, water and sanitation facilities, and access to and personal medical beliefs on health care. Height and weight were measured. Body mass index (BMI) was calculated and weight categorized according to BMI for age. Otoscopy was performed on both ears, and each ear was described for the presence and character of perforation or discharge. If the 2 ears from the same individual had different diagnoses, otitis media status was determined according to the worse ear. Acute otitis media, usually <2 weeks, was defined as an episode of ear perforation with discharge or as an intact but bulging eardrum with hyperemia and pain. Effusive otitis media was diagnosed for nonhyperemic intact tympanic membranes with dullness, retracted position, or poor movement with insufflation, with or without visible fluid behind the eardrum. Otitis media was considered chronic if persisting >3 months. Eardrums with signs of previous perforation or scarring but with no current perforation, discharge, hyperemia, or fluid behind the eardrum were noted to have healed otitis media. For ears that were examined multiple times over the years, persistence, recurrence, and resolution of otitis media were also noted, with the last ear examination as the basis of final diagnosis.

For assessment of the relation of different variables to otitis media status, standard statistical testing was performed with R.13 Fisher exact tests were performed to see the relation of otitis media status with carriage of the A2ML1 variant, age strata, sex, breastfeeding, BMI categories, tobacco exposure, and deep swimming. Logistic regression was done to determine if otitis media status is dependent on BMI and age as continuous variables. For multivariate analysis, logistic regression was performed, with otitis media status as the dependent variable and 3 independent variables with \( P < .2 \) in bivariate analyses (ie, A2ML1 variant, household membership, interaction between age and sex).

### Results

A total of 187 community members were examined by otoscopy, and 91 had current or previous otitis media (Table 1). Chronic otitis media was diagnosed in 37 individuals with eardrum perforations, mostly with mucoid discharge. Acute otitis media was identified in 13 patients and effusive otitis media in 10. Of those with perforated ears at last examination, about half were bilaterally affected. An additional 31 recovered from active otitis media, had healed perforations, or had thickened/scarred eardrums. Young age was associated with otitis media if divided into 4 strata (Fisher’s exact, \( P = .004 \)); however, if age had only 2 strata (threshold, 12.5 years) or was treated as a continuous variable, it was non-significant. There was no association between otitis media and sex, low or high BMI, breastfeeding in infancy, tobacco exposure, or deep swimming. However, for some of these variables, information was collected in only 50% to 80% of participants, and nonsignificance may be due to small sample sizes.

The indigenous Filipino population was identified to have a 48.7% prevalence of otitis media (Figure 1). Based on interviews of the heads of 25 households, environmental backgrounds were relatively homogeneous, with low incomes (average US$40 per month) and low educational

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**Table 1. Description of Indigenous Filipinos with or without Otitis Media.**

<table>
<thead>
<tr>
<th>Variable</th>
<th>n (%)</th>
<th>Variable</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Population size (~2014)</td>
<td>200</td>
<td>Body mass index</td>
<td>150 (80.2)</td>
</tr>
<tr>
<td>Individuals examined</td>
<td>187 (93.5)</td>
<td>Underweight</td>
<td>40 (26.7)</td>
</tr>
<tr>
<td>Individuals with otitis media</td>
<td>91 (48.7)</td>
<td>Overweight</td>
<td>30 (20.0)</td>
</tr>
<tr>
<td>Chronic</td>
<td>37 (40.7)</td>
<td>Breastfed (n = 127)</td>
<td>104 (81.9)</td>
</tr>
<tr>
<td>Acute</td>
<td>13 (14.3)</td>
<td>Tobacco exposure (n = 118)</td>
<td>77 (65.3)</td>
</tr>
<tr>
<td>Effusive</td>
<td>10 (11.0)</td>
<td>Swimming in deep water (n = 92)</td>
<td>18 (19.6)</td>
</tr>
<tr>
<td>Healed</td>
<td>31 (34.1)</td>
<td>DNA samples obtained</td>
<td>85 (45.5)</td>
</tr>
<tr>
<td>Perforated ears</td>
<td>58</td>
<td>A2ML1-carrier with otitis media</td>
<td>50 (58.8)</td>
</tr>
<tr>
<td>Bilateral perforations</td>
<td>19</td>
<td>Wild type</td>
<td>35 (41.2)</td>
</tr>
<tr>
<td>Age at last examination, y</td>
<td></td>
<td>Wild type with otitis media</td>
<td>17 (48.6)</td>
</tr>
<tr>
<td>&lt;5</td>
<td>41 (21.9)</td>
<td>With ear swabs/microbiome data</td>
<td>16</td>
</tr>
<tr>
<td>5-12</td>
<td>57 (30.5)</td>
<td>A2ML1-variant carrier with swabs</td>
<td>11</td>
</tr>
<tr>
<td>13-25</td>
<td>41 (21.9)</td>
<td>Total households (2006 survey)</td>
<td>43</td>
</tr>
<tr>
<td>&gt;25</td>
<td>48 (25.7)</td>
<td>Average household members (2006)</td>
<td>5</td>
</tr>
</tbody>
</table>
attainments (most families do not have a high school graduate). In 2006, >90% of families lived in huts, and cooking was mostly done with firewood. Inhabitants had access to piped water but poor sanitary facilities, with 3 families on average sharing 1 toilet. When asked about beliefs regarding ear discharge, 56% of respondents believed that it is normal for children to have ear discharge, while 16% answered “maybe.” When asked if it is normal for a child to be hearing impaired, 28% answered “yes,” 16% “a little bit,” and 12% “maybe.” A quarter of respondents never sought consult for ear symptoms, while half sometimes sought clinical consult, with reasons cited being ear discharge, ear pain, and access to medical missions in the area. Medical personnel and facilities are available on the island, but inhabitants’ personal medical beliefs reflect a lack of public health awareness about otitis media that, coupled with low income, results in a decreased personal value for continued medical care. In 2012, the community was granted a 2-hectare plot of protected land. The latest visit in 2014 revealed improvements in living conditions, including mixed brick-and-wood dwellings, a flush toilet for each family, stoves for cooking, and full access to piped water. Interestingly, rates of healed otitis media slightly increased in 2014 versus previous years, although overall otitis media prevalence was stable at 46.3% to 49.1% from 2010 to 2014 (Figure 1).

Previously, the A2ML1 c.2478_2485dupGGCTAAAT p.(Ser829Trpfs*9) duplication variant was shown to be associated with otitis media through 2 independent study groups—namely, a large pedigree within the indigenous Filipino population and a case-control cohort of US children. Aside from 51 indigenous Filipinos who provided DNA samples in the original study, in this report an additional 34 participants provided DNA samples. Of 85 patients who provided DNA samples, 50 (58.8%) were either heterozygous or homozygous for the A2ML1 variant, while the rest were wild type (Table 1). When multivariate regression was performed with the A2ML1 genotype, household membership, and the interaction between age and sex as independent variables, only carriage of the A2ML1 variant was a significant predictor of otitis media occurrence (P = .005). In addition, 80% of those who carry the A2ML1 variant developed otitis media, while less than half of those who are wild type had current or previous otitis media (Table 1). The odds ratio for otitis media given the A2ML1 genotype was 3.7 (95% confidence interval: 1.3-10.8; P = .005).

When all ear diagnoses were plotted within an age continuum (Figure 2A), diagnoses of otitis media started within the first months of life. The peak age for chronic otitis media was at 10 years, with a second peak at ages 2 to 5. Acute otitis media was more common at 0 to 4 years and effusive otitis media at 5 to 6 years. At 2 to 5 years, indigenous Filipino children may experience recurrent acute/effusive otitis media or proceed to chronic otitis media; then, at ages 6 to 12, they either continue with chronic otitis media or heal. From teenagers to adults, the proportion of active otitis media progressively decreases. In terms of A2ML1-variant carriers versus wild type, carriers seem to have an earlier age of otitis media onset at <1 year old,
with peak ages of active otitis media at 6 and 10 years (Figure 2B). In $A2ML1$-variant carriers, otitis media kept occurring up to ~30 years, with 81.6% of examinations revealing active or healed otitis media rather than normal otoscopic findings (Figure 2B). In contrast, there is much less occurrence of active or healed otitis media (57.1%) in wild-type individuals, with more normal examinations in infancy and in those $>$13 years old (Figure 2C). Overall, these findings may imply that carriage of the $A2ML1$ variant affects otitis media onset and recovery.

Our microbiome studies showed that (1) Oligella and Corynebacterium were abundant in both $A2ML1$-variant carriers and noncarriers; (2) the phyla Bacteroidetes and Fusobacteria and genera Porphyromonas and Fusobacterium were more abundant in $A2ML1$-variant carriers; (3) Proteobacteria at the phylum level and Haemophilus at the genus level had higher relative abundance in wild-type individuals; and (4) GKS98 freshwater bacteria, which was previously reported in environmental samples only, was also detected in the middle ears of indigenous Filipinos with chronic otitis media. When people with middle ear swabs were grouped according to $A2ML1$ genotype, age, sex, and household, there seemed to be clustering of specific bacteria based on age and/or sex (Figure 3). All bacteria were detected at $\geq5\%$ abundance within the middle ear. Patients with bilateral otitis media (Nos. 5, 33, 53, 63, 92) had similar abundance levels of bacteria on both ears. The assumption is that male and female children play with each other and consist of a social cluster. Teenage and older females are also clustered. Two caveats here are as follows: (1) the sample size is limited by the occurrence of eardrum perforations; and (2) diagnosis of chronic otitis media peaks at 5 to 10 years, thus, more than half of those with microbial samples are within this age group (Figure 3). Taken together, these findings might imply that although the $A2ML1$ genotype is the primary risk factor for otitis media diagnosis and the determinant of disease progression, age and social clusters (eg, household membership, cluster by age and sex) may modulate the effect of the $A2ML1$ genotype on carriage of specific bacterial taxa within the middle ear.

**Discussion**

Of all the variables studied, the $A2ML1$ genotype is the strongest predictor of otitis media within the indigenous Filipino population, increasing the risk of all forms of otitis media in $A2ML1$-variant carriers almost fourfold. Although age and household membership were not significant in multivariate analysis, there is indication that age and social clusters both play a role in carriage of specific bacterial taxa according to the $A2ML1$ genotype. Additionally, onset of and recovery from otitis media were different in $A2ML1$-
variant carriers versus noncarriers and were highly dependent on age. The main strength of the study is the relative homogeneity of environmental backgrounds within the indigenous Filipino community, which allows for study of otitis media according to only a few variables. Nevertheless, this study shows that in spite of the strong association between the A2ML1 variant and otitis media, A2ML1-related otitis media remains a complex trait shaped by multiple factors, including age, social clusters, and middle ear bacteria.

In our previous study, we identified abundant middle ear bacteria according to the A2ML1 genotype as well as bacteria that were rarely or never reported in the middle ear—namely, Oligella and GKS98 freshwater bacteria. Among the top bacterial genera identified, Oligella and Corynebacterium were abundant in A2ML1-variant carriers and noncarriers alike (Figure 3). Corynebacterium is commonly isolated from middle ear fluid cultures, but Oligella was reported only once in ear discharge. In wild-type individuals, Proteobacteria at the phylum level and Haemophilus at the genus level were more abundant as compared with A2ML1-variant carriers. Nontypeable Haemophilus influenzae causes acute otitis media, especially in indigenous children, so this finding is not surprising. In contrast, the phyla Bacteroidetes and Fusobacteria and genera Porphyromonas and Fusobacterium were more abundant in A2ML1-variant carriers (Figure 3). Although these 2 genera were previously isolated from the culture of ear discharge with chronic otitis media, they are much less common than Proteus, Pseudomonas, and Staphylococcus, which are usually isolated in chronic otitis media patients worldwide and in the Philippines. The uniqueness of the microbial ear profiles of indigenous Filipinos can be attributed to the occurrence of the A2ML1 variant and probably to the social structures within the community.

The diagnosis of otitis media in indigenous Filipino infants is consistent with findings in indigenous Australian children in whom acute otitis media was observed within the first 3 months of life. While indigenous Australian children have mostly acute otitis media, indigenous Filipino children have chronic otitis media as early as 2 years of age, particularly among A2ML1-variant carriers (Figure 2A). Carriers of the A2ML1 variant appear to have not just an earlier onset but also a more protracted course of otitis media (Figure 2B). Some wild-type individuals do have persistently chronic otitis media beyond 13 years old (Figure 2C), and for these specific participants, the possibility of a second otitis media susceptibility variant is currently being investigated. Thus, among indigenous populations where socioeconomic background may favor an increase in prevalence of otitis media, the indigenous Filipino population is unique in having at least 1 rare mutation that confers susceptibility to otitis media, favors carriage of specific middle ear pathogens, and influences disease patterns, including chronicity and onset.

Whether less crowded conditions and better sanitation within the indigenous Filipino community are related to changes in rates of otitis media is unknown. There is, however, some indication of an increase in rates of healed otitis media after improvement in living conditions, and longer surveillance should help elucidate if better hygiene alleviates the burden of otitis media within the community. Additionally a more complete survey on A2ML1 genotype, middle ear bacteria, and household/social clusters should further illuminate the contribution of these factors to the increased prevalence of otitis media within the community. More important, the lack of access to health care is a systemic issue that needs to be addressed through advocacy. The PHiD-CV10 vaccine confers additional protection against nontypeable H influenzae as compared with other pneumococcal vaccines to which a decrease of otitis media incidence has been attributed. The PHiD-CV10 vaccine was recently included in the Philippine immunization program, and to our knowledge at the time of study, none of the indigenous community members were vaccinated with it or any pneumococcal vaccine. Through scientific study of the interplay of genetic and environmental factors that lead to otitis media, it is our hope that these results may be useful in supporting special appropriations toward the institution and maintenance of public health measures (eg, antibiotic and surgical treatment, vaccination) for otitis media within the indigenous Filipino community.

Taken together, our findings suggest that among indigenous Filipinos, the A2ML1 genotype is the primary risk factor for otitis media and the main determinant of disease progression, although age, the middle ear microbiome, and social clusters might modulate the effect of the A2ML1 genotype.

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Author Contributions
Reggie Lyn P. Santos-Cortez, conception of study design, data collection, analysis and interpretation, manuscript writing, final approval, accountable for all aspects of the work; Ma. Rina T. Reyes-Quintos, conception of study design, data collection, manuscript writing, final approval, accountable for all aspects of the work; Ma. Leah C. Tantoco, data collection, manuscript writing, final approval, accountable for all aspects of the work; Izoduwa Abbe, data collection, manuscript writing, final approval, accountable for all aspects of the work; Erasmo Gonzalez d.V. Llanes, data collection, manuscript writing, final approval, accountable for all aspects of the work; Nadim J. Ajami, data analysis and interpretation, manuscript writing, final approval, accountable for all aspects of the work; Diane S. Hutchinson, data analysis and interpretation, manuscript writing, final approval, accountable for all aspects of the work; Joseph F. Petrosino, data analysis and interpretation, manuscript writing, final approval, accountable for all aspects of the work; Carmencita D. Padilla, conception of study design, manuscript writing, final approval, accountable for all aspects of the work; Tantoco, conception of study design, data collection, analysis and interpretation, manuscript writing, final approval, accountable for all aspects of the work; Santos-Cortez, conception of study design, data collection, analysis and interpretation, manuscript writing, final approval, accountable for all aspects of the work; Padilla, conception of study design, manuscript writing, final approval, accountable for all aspects of the work; Garcia, P. J. Labra, K. Fellizar, D. Roldan, C. Espina, D. Vanguardia, M. Pedro, S. M. Lagrina, and V. Ostan. We are grateful to Tulin Ayvaz from the CMMR for her work in sample processing and to Ginger Metcalfe, Donna Muzny, and Richard Gibbs from the Human Genome Sequencing Center at Baylor College of Medicine for their support in sequencing.
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