Rare A2ML1 variants confer susceptibility to otitis media

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A duplication variant within the middle ear–specific gene A2ML1 cosegregates with otitis media in an indigenous Filipino pedigree (LOD score = 7.5 at reduced penetrance) and lies within a founder haplotype that is also shared by 3 otitis-prone European-American and Hispanic-American children but is absent in non-otitis-prone children and >62,000 next-generation sequences. We identified seven additional A2ML1 variants in six otitis-prone children. Collectively, our studies support a role for A2ML1 in the pathophysiology of otitis media.

Otitis media causes considerable morbidity worldwide and hearing loss at any age. Despite efforts to reduce its incidence, otitis media remains an important public health problem within the United States, with otitis media being the most frequent cause of pediatric consults and antibiotic prescription, incurring an annual cost of >$5 billion. In developing countries, including the Philippines, the prevalence of chronic suppurative otitis media is 2–6% (ref. 2). Strong evidence exists for genetic susceptibility to otitis media but is absent in non-otitis-prone children and >62,000 next-generation sequences. We identified seven additional A2ML1 variants in six otitis-prone children. Collectively, our studies support a role for A2ML1 in the pathophysiology of otitis media.

We obtained DNA samples from 123 otitis-prone and 118 non-otitis-prone children who were followed from birth at the University of Texas Medical Branch (UTMB). Among the UTMB children, 84 (68.3%) otitis-prone and 79 (66.9%) non-otitis-prone children
self-identified as American or Hispanic American. Sanger sequencing of all A2ML1 coding exons showed that the same A2ML1 duplication was present in 3 of 123 otitis-prone children. Two otitis-prone children, one European American and the other Hispanic American, were homozygous for the duplication, whereas a third otitis-prone, European-American child was heterozygous (Table 1 and Supplementary Table 2). We verified the reported ancestry for these three otitis-prone carriers by principal-components analysis (Supplementary Fig. 2). All three children with the duplication had early-onset severe otitis media requiring tympanostomy tube insertion by 6 months of age. Additionally, the duplication was absent in the 118 non-otitis-prone children (Supplementary Table 2), 2,756 UWCMG chromosomes of European-American or Hispanic-American descent (Supplementary Fig. 1), and 67,630 European, non-Finnish and 11,606 Latino alleles from the ExAC database (Table 1). Comparing the frequency of this duplication only in individuals of European-American or Hispanic-American descent, we found that the duplication had genome-wide significant association with otitis media (two-sided Fisher’s exact test, \( P = 3.34 \times 10^{-14} \)). Moreover, the two exome-sequenced indigenous individuals and three otitis-prone children shared a haplotype that included the duplication and three common variants (Supplementary Table 2). The A-dup-A-T haplotype included 5.2 kb and is estimated to be \( \sim 1,800 \) years old (95% confidence interval = 145–3,462 years). This short founder haplotype was most likely introduced to the Americas and the Philippines by colonial Spaniards, according to population history.

Seven additional variants (three stop-gain and four missense) were each identified as heterozygous in an otitis-prone child but not in non-otitis-prone children (Table 1). With the exception of the A2ML1 duplication, all additional variants identified in the UTMB cohort each occurred in a single proband. All seven single-nucleotide variants identified in otitis-prone children from UTMB occurred at highly conserved nucleotides, were predicted to be damaging, had scaled Combined Annotation-Dependent Depletion (CADD) score >15 (ref. 12), and were absent in UWCMG exomes and SSMP. Five of the seven variants were not in \( \geq 121,144 \) alleles in the ExAC database (Table 1). Owing to the extremely low frequency of these variants, when they were tested for association by comparing their frequencies in the otitis-prone children to those in European-American or Hispanic-American individuals in the non-otitis-prone UTMB cohort, UWCMG and ExAC, we found that, although none of these variants were associated with otitis media at a genome-wide significance level, all were nominally significant (two-sided Fisher’s exact test, \( P < 0.05 \); Table 1). One Hispanic-American otitis-prone child was heterozygous for both a stop-gain mutation, c.2914G>T (p.Glu972*), and a missense mutation, c.955G>A (p.Ala810Thr). Molecular modeling for these two variants predicted domain loss due to the stop-gain variant but no obvious changes due to the missense variant (Supplementary Fig. 3); thus, it is possible that only the p.Glu972* variant contributes to otitis media susceptibility. Among five other variants in UTMB otitis-prone children, the stop-gain variants p.Gln255* and p.Arg893* were likewise predicted to result in domain loss; in contrast, of the missense variants, only p.Pro356Arg and p.Arg1001Trp were shown by molecular modeling to cause torsional changes and loss of hydrogen bonds (Supplementary Fig. 3).

Furthermore, 622 DNA samples from 143 families ascertained at the University of Minnesota (UMN) were available for study. In European-American family 123, a missense variant, c.887T>C (p.Val296 Ala), was heterozygous in two siblings and their mother, all three of whom had documented otorrhea, eardrum perforation or abnormalities, and/or previous or current otitis media. The unaffected father and a third sibling who had a single prolonged episode of otitis media but no eardrum abnormalities were wild type for the variant. The missense variant had scaled CADD score = 15.2, was at a conserved nucleotide and predicted to be damaging by two of six algorithms, and occurred in a domain that is rich in disulfide bonds, which may be needed to link A2ML1 monomers into the active dimer and tetramer formations\(^\text{10}\). However, this variant was heterozygous in 96 alleles from the ExAC database (European, non-Finnish minor allele frequency = 0.0013) and probably does not confer otitis media susceptibility.

We assessed the middle ear localization of A2ML1 using high-resolution confocal imaging in mice. At postnatal day (P) 6, we observed immunoreactivity for \( \beta \)-catenin (positive control) and A2ML1 in mucosal epithelium (Fig. 2). In contrast, A2ML1 staining was almost absent in mouse inner ear (Supplementary Fig. 4), which is consistent with no detectable A2ML1 expression within the Shared Harvard Inner-Ear Laboratory Database.

The similarity of A2ML1 to A2M suggests that these protease inhibitors might have similar and overlapping protective functions within the middle ear. A2M is detected at low levels in serous middle ear effusions, but its levels are increased in acute purulent otitis media, with a greater proportion found in complex with proteases, indicating leakage into the middle ear of high-molecular-weight A2M owing to increased mucosal permeability and greater inhibitory A2M activity during infection\(^\text{8,13}\).

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**Figure 1** Segregation within the indigenous pedigree, schematic of A2ML1 domains and molecular modeling for the A2ML1 variant. (a) Pedigree connecting 37 variant carriers who have the full spectrum of otitis media (OM; age 3 months to 58 years, median of 13 years). An individual with healed otitis media and intellectual disability is wild type for the duplication, but her 13-year-old son who has chronic otitis media but no intellectual disability and her unaffected 4-month-old daughter are heterozygous. Nine variant carriers have no evidence of otitis media (median age of 18 years), whereas four individuals are wild type and unaffected. (b) Predicted A2ML1 domains based on the α2-macroglobulin structure\(^\text{10}\). MG, macroglobulin-like domains 1–7; BRD, bait region domain; CUB, consists of two four-stranded antiparallel β sheets; TED, thiol-ester domain; RBD, receptor-binding domain. The A2ML1 frameshift variant is expected to occur within the MG7 domain (red arrow). (c) Modeling predicts loss of the receptor-binding and thiol-ester domains due to the A2ML1 duplication.
A2ML1 variants identified in otitis-prone children from UTMB

| CHS 12q14.3-14.2 | Proximal Del. | ExAC MAF | UTMB MAF | Fisher’s exact test P value | ExAC MAF | UTMB MAF | Fisher’s exact test P value |
|------------------|---------------|----------|----------|-----------------------------|----------|----------|-----------------------------|
| GRPP             |              |          |          |                             |          |          |                             |
| 8,998,070        | 1.4          | 1/67,250 | 0/11,464 | 0.001                       | 1/67,250 | 0/11,464 | 0.001                       |
| 8,998,086        | 1.9          | 1/67,250 | 0/11,464 | 0.001                       | 1/67,250 | 0/11,464 | 0.001                       |
| 8,998,573        | 2.2          | 1/67,250 | 0/11,464 | 0.001                       | 1/67,250 | 0/11,464 | 0.001                       |
| 9,004,827        | 1.3          | 1/67,250 | 0/11,464 | 0.001                       | 1/67,250 | 0/11,464 | 0.001                       |
| 9,006,810        | −1.0         | 1/67,250 | 0/11,464 | 0.001                       | 1/67,250 | 0/11,464 | 0.001                       |
| 9,008,582        | 2.8          | 1/67,250 | 0/11,464 | 0.001                       | 1/67,250 | 0/11,464 | 0.001                       |
| 9,009,091        | 3.4          | 1/67,250 | 0/11,464 | 0.001                       | 1/67,250 | 0/11,464 | 0.001                       |
| 9,009,301        | 0.0          | 1/67,250 | 0/11,464 | 0.001                       | 1/67,250 | 0/11,464 | 0.001                       |
| 9,010,291        | 2.7          | 1/67,250 | 0/11,464 | 0.001                       | 1/67,250 | 0/11,464 | 0.001                       |
| 9,004,827        | −1.0         | 1/67,250 | 0/11,464 | 0.001                       | 1/67,250 | 0/11,464 | 0.001                       |
| 9,006,810        | −1.0         | 1/67,250 | 0/11,464 | 0.001                       | 1/67,250 | 0/11,464 | 0.001                       |
| 9,008,582        | 2.8          | 1/67,250 | 0/11,464 | 0.001                       | 1/67,250 | 0/11,464 | 0.001                       |
| 9,009,091        | 3.4          | 1/67,250 | 0/11,464 | 0.001                       | 1/67,250 | 0/11,464 | 0.001                       |
| 9,009,301        | 0.0          | 1/67,250 | 0/11,464 | 0.001                       | 1/67,250 | 0/11,464 | 0.001                       |
| 9,010,291        | 2.7          | 1/67,250 | 0/11,464 | 0.001                       | 1/67,250 | 0/11,464 | 0.001                       |

| Variant          | Type            | Coverage | Ancestry prediction | PhyloP100 Score | GERP G Score | Combined Annotation-Dependent Depletion (CADD) | DANscore | SeattleSeq Annotation | dbNSFP |
|------------------|-----------------|----------|---------------------|----------------|--------------|-------------------------------------------------|----------|-----------------------|--------|
| c.955G>A         | Missense        | 15.1     | MA, PP2, S          | 110            | 100          | 0.001                                           | = 0.0005 | = 0.0001              | = 0.0001|
| c.2914G>T        | Stop gain       | 36.0     | MT                  | 158            | 168          | 0.002                                           | = 0.0005 | = 0.0001              | = 0.0001|

UTMB 1031 is heterozygous for two variants, c.955G>A (p.Ala810Thr) and c.2914G>T (p.Glu972*). All listed variants were not identified in non-otitis-prone UTMB children, 1,385 UWCMG exome sequences and 100 genomes from SSMP. Two-sided Fisher’s exact tests were performed by comparing the variant frequency in UTMB otitis-prone children of European-American or Hispanic-American descent with the combined frequency in UTMB non-otitis-prone children of European- or Hispanic-American descent, 1,378 UWCMG exomes of European- or Hispanic-American descent, and European, non-Finnish and Latino alleles in ExAC. For variants that were not identified in ExAC, the number of individuals carrying each allele is based on this total number of alleles screened for the variant site listed, coverage in ExAC was 50–95× on average and at least 3×o in 90–95% of 90× samples.

Additionally, low serum A2M levels were detected in children with recurrent otitis media. The tetrameric A2M structure is an effective trap for proteases, which if left unchecked can damage middle ear mucosa. Using A2M as a template, A2ML1 variants are predicted to affect thiol protease trapping, tetramer formation and/or receptor-mediated clearance of A2ML1-protease complexes. Interestingly, bacitracin, which is an antibiotic component of eardrops used in Europe for acute otitis media with otitis media, competitively inhibits binding of A2M-protease complexes to macrophages or fibroblasts for clearance. If bacitracin dampens A2M activity, which might compensate for dysfunctional A2ML1, it can be hypothesized that, for A2ML1 variant carriers, bacitracin might not be the antibiotic of choice for otitis media.

A2ML1 variants were previously found in Noonan-like syndrome cases; however, otitis media was not described in these cases, and it was not mentioned whether the hearing loss in two A2ML1 variant carriers with Noonan-like syndrome was conductive or sensorineural. The A2ML1 carriers reported here do not have cardinal features of Noonan syndrome. We therefore present a rare genetic cause of susceptibility to non-syndromic otitis media, which affects a total of 37 indigenous Filipino, 1 Hispanic-American and 2 European-American individuals. A2ML1 localizes specifically to middle ear epithelium, thus supporting a role in the pathophysiology of otitis media.

METHODS

Methods and any associated references are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

R.L.P.-S. and S.M.L. conceptualized the study, R.L.P.-S., C.M.C., M.R.T.R.-Q., M.L.C.T., M.C.G., E.G.D.V.L., P.J.L., T.L.I.G.-C., A.L.C., E.M.C.-d.l.P. and G.T.A. provided clinical data and DNA samples from the indigenous population, J.A.P. and T.C. collected data and DNA samples from the UTMB cohort, E.K.A., K.A.D. and M.M.S. provided clinical data and DNA samples from the UMN cohort, X.W., A.A., I.A. and R.L.P.-S. performed DNA isolation and PCR sequencing. The UWMCG, J.D.S., J.S., M.J.B. and D.A.N. performed genotyping and exome sequencing and provided exome data from non-otitis samples, J.D.S. performed principal-components analysis. G.T.W. and R.L.P.-S.-C. performed exome analysis.

URLs

Combined Annotation-Dependent Depletion (CADD), http://cadd.gs.washington.edu/; dbNSFP, https://sites.google.com/site/jpogen/dbNSFP; dbSNP, http://www.ncbi.nlm.nih.gov/SNP/; Exome Aggregation Consortium (ExAC), http://exac.broadinstitute.org/; Genome Analysis Toolkit (GATK), https://www.broadinstitute.org/gatk/; Protein Data Bank, http://www.rcsb.org/pdb/home/home.do; SeattleSeq Annotation, http://snp.gs.washington.edu/SeattleSeqAnnotationI37/; Shared Harvard Inner-Ear Laboratory Database, https://shield.hms.harvard.edu/; UCSC Genome Bioinformatics, http://genome.ucsc.edu/; US Census Bureau Population Estimates, http://www.census.gov/popest/; Variant Mendelian Tools, http://varianttools.sf.net/VMT.
B.L., estimated haplotype age. A.P.G., S.R., and Z.M.A. performed immunolocalization experiments. R.L.P.S.-C., S.R., Z.M.A., and S.M.L. wrote the manuscript. All authors read, provided critical input and approved the final version of the manuscript.

COMPETING FINANCIAL INTERESTS
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Figure 2 A2ML1 is localized to middle ear epithelium. (a, c) Sagittal cryosections from P6 wild-type mice showing the middle ear cavity (MEC), middle ear mucosa (MEM) and semicircular canal of the inner ear (SCC). (b, d) Higher magnification depicting the boxed sections in a and c. (a–d) Confocal images of the cryosections stained with DAPI (blue) and immunostained with antibodies for A2ML1 (green in a and b), β-catenin (green in c and d) and rhodamine phalloidin (actin; red). White arrowheads point to MEM with A2ML1 (b) and β-catenin (d) expression. DIC, differential interference contrast. Scale bars, 100 μm.
Subject ascertainment. The indigenous population comprises the original inhabitants of an island within the central Philippines and is of Negrito stock, which is historically known to consist of the first wave of migrants to the Philippines 20,000–30,000 years ago. The indigenous peoples in the same region had early contacts with colonial Spaniards in the late sixteenth century, and some of the earliest Spanish settlements were built on the main island. Owing to characteristic Negrito features such as short stature, curly hair, flat nose and dark skin, the indigenous population has been subjected to racial and socioeconomic discrimination over the centuries, which fostered intermarriage within the same community. Genetic comparison with other populations shows that this indigenous population has multiple elements of admixture but remains primarily of mixed Negrito and Indo-Malay ancestry.\textsuperscript{18}

Of a current population of ~250 indigenous individuals, we performed otoscopy on 175 members of the indigenous community, of whom 82 (46.9\%) were currently or previously diagnosed with otitis media. A pedigree that connects 134 indigenous individuals, of whom 51 provided DNA samples, was constructed (Fig. 1a). For the indigenous pedigree, diagnosis was based on the following otoscopic findings: chronic otitis media, eardrum perforation with well-defined borders, usually with thickened middle ear mucosa and mucopurulent discharge; acute otitis media, hyperemic eardrum with or without perforation or discharge; otitis media with effusion, dull or retracted, intact, non-hyperemic eardrum with middle ear fluid; and intact non-hyperemic eardrum with healed perforation or scarring and/or previously documented otitis media. None of the ascertained individuals had craniofacial deformities, cardiopulmonary defects or immunodeficiency that would cause recurrent infections. No dermatological diseases or intellectual disabilities cosegregated with otitis media. Saliva was collected from 38 affected and 13 unaffected pedigree members, and DNA was extracted using Oragene kits (DNA Genotek).

To replicate study findings from the indigenous population, DNA samples were obtained from 2 previously established otitis media cohorts: (i) a case-control cohort from UTMB that included 123 otitis-prone and 118 non-otitis-prone children\textsuperscript{5} and (ii) a family cohort from UMN that consisted of 622 DNA samples from 143 families\textsuperscript{6}. For the UTMB cohort, children were considered otitis prone on the basis of any of the following criteria: first episode of acute otitis media at <6 months; ≥3 episodes of acute otitis media within a 6-month period; ≥4 episodes of acute otitis media within a 12-month period; ≥6 episodes by 6 years of age; or tympanostomy tube placement for recurrent or persistent otitis media.\textsuperscript{5} Non-otitis-prone children included those with 0–2 episodes of acute otitis media by 2 years of age. Children were excluded from study if they had ear or nasopharyngeal defects, immunological abnormalities or major medical conditions or if they were undergoing treatment for chronic diseases.

At UMN, each family (median size of four with two affected individuals) was recruited when the proband underwent tympanostomy tube insertion for chronic or recurrent otitis media.\textsuperscript{6} All family members were examined by an otolaryngologist and tested by tympanometry. Family members were considered affected if ≥2 data sources, whether otoscopy, tympanometry, medical records or personal history, were positive for otitis media. Family members and probands who had Down syndrome, craniofacial anomalies, genetic syndromes with otitis media or tympanostomy tubes not due to otitis media were excluded from study.

DNA sequencing and genotyping. Exome sequencing was performed on the UWCMG on an Illumina HiSeq instrument, sequencing to an average read depth of ~60×. Sequence capture was performed using the Roche NimbleGen Big Exome 2011 Library. Sequence alignment and variant detection, calling and annotation were performed as described\textsuperscript{19}. Selected variants that were observed as heterozygous in both indigenous Filipino exomes (Supplementary Table 1) underwent Sanger sequencing using 51 DNA samples from the indigenous Filipino pedigree. All coding exons of A2ML1 were sequenced using DNA for 123 otitis-prone children from the UTMB cohort and 143 probands from UMN families. Exons containing variants were also sequenced using samples from 4 members of UMN family 123 (exon 9) and 118 non-otitis-prone children from UTMB (exons 8, 9, 10, 19, 20, 21, 24 and 34).

The occurrence of 9 A2ML1 variants was also checked in exome sequence data from 1,385 unrelated individuals whose DNA samples were submitted to UWCMG for causal gene identification for various phenotypes (Supplementary Fig. 1). None of these individuals were reported to have otitis media. Ancestry was inferred for these individuals using principal-components analysis.

To verify the ancestry of the UTMB children, DNA samples from 3 otitis-prone children who carried the A2ML1 duplication variant as well as 38 non-otitis-prone children with sufficient DNA content were submitted to UWCMG for genotyping using the Illumina HumanCoreExome-24v1 beadChip, which includes ~540,000 markers. The generated genotypes were used to perform principal-components analysis with comparison to HapMap 3 genotypes (Supplementary Fig. 2).

Bioinformatic, linkage and haplotype analyses. For variant identification from exome data, sequences were analyzed using Variant Mendelian Tools, a modification of Variant Association Tools\textsuperscript{20}, which facilitates variant selection on the basis of sharing among relatives and annotation from RefSeq, dbSNP, SeattleSeq, ExAC, CADD and dbNSFP, among others. The dbNSFP database includes nucleotide conservation scores (GERP and phyloP) and prediction for nonsynonymous variants from six different algorithms (fathmm, likelihood-ratio test, MutationAssessor, MutationTaster, PolyPhen-2 and SIFT). Shared nonsynonymous variants in the two indigenous Filipino exomes were selected for further study if they passed GATK filters (ABFilter, HRunFilter, QDFilter, QUALFilter, SBFilter and SnpCluster); were absent from dbSNP and the ExAC database; occurred at conserved nucleotides; had scaled CADD scores ≥15; and were predicted to be damaging by ≥3 algorithms. Because of database limitations for indel annotation, shared indel variants within coding regions that passed GATK filters were kept under consideration.

For the six variants identified from exome sequence data, two-point linkage analysis was performed for the indigenous Filipino pedigree using Superlink\textsuperscript{21} (Supplementary Table 1). All individuals with chronic otitis media, acute otitis media, otitis media with effusion or healed otitis media were considered to be affected. The penetrance is unknown for otitis media susceptibility variants. In addition, adults with normal otoscopic findings may have had undocumented otitis media in childhood or some individuals might have recurrent otitis media but be unaffected at the time of examination. Therefore, affected-only linkage analysis, in which all unaffected pedigree members were assumed to have an unknown affected status, was performed using an autosomal dominant mode of inheritance and a disease allele frequency of 0.0001. Affected-only analysis was performed under two conditions: (i) full penetrance with no phenocopies and (ii) 95% penetrance with a 5% phenocopy rate (Supplementary Table 1).

Molecular modeling for A2ML1 variants was performed using Phyre2 (ref. 22) with the human A2M structure as the template (Protein Data Bank (PDB), 4ACQ). The age of the haplotype that included the A2ML1 duplication was estimated using DMLE+ (ref. 23). Genotypes from 241 unrelated UTMB children for 3 SNPs surrounding the A2ML1 variant (rs1860927, rs1860926 and rs78452682) were included in the analysis (Supplementary Table 2). A population growth rate of 1.2%, based on annual US estimates, was used. Galveston Island, Texas, has a population of ~48,000, and the proportion of otitis-prone children among all children followed from birth is 4.4%. Thus, the proportion of the population sampled was specified to be 0.001 in the analysis. Each generation was assumed to occur at 25-year intervals.

Immunolocalization studies. Six wild-type C57BL/6J mice (three male and three female) were used. For the middle ear, hemi-dissected heads of P6 mice.
were fixed overnight at 4 °C with 4% paraformaldehyde (PFA) and were incubated first in a solution of 0.25 M EDTA in 1× PBS for 24 h and then in a solution of 30% sucrose in 1× PBS for 4 h. Samples were embedded in OCT, and 14-μm sections were cut using a cryostat. For the organ of Corti, P7 mice were euthanized and decapitated. Temporal bones were isolated and fixed overnight at 4 °C with 4% PFA, and fine dissection of the cochleae was performed to isolate organs of Corti.

Sections were permeabilized with PBS containing 0.25% Triton X-100 for 1 h and blocked with 10% normal goat serum (Vector Labs) diluted in PBS for 1 h. Sections were incubated overnight at 4 °C with antibody to A2ML1 (Abcam, ab72872; 1:200 dilution in 3% normal goat serum in PBS) or, for middle ear sections, with antibody to β-catenin (Santa Cruz Biotechnology, sc-7199; 1:200 dilution) and then washed and incubated with a secondary antibody in 3% normal goat serum in PBS for 1 h at room temperature. Rhodamine phalloidin was used at a 1:300 dilution for F-actin labeling (Invitrogen). Nuclei were stained with DAPI (Molecular Probes). Samples were mounted using Prolong Gold Antifade mounting medium (Molecular Probes) and imaged using a 100× objective on a confocal microscope (LSM 700, Carl Zeiss).

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