Association between earwax-determinant genotypes and acquired middle ear cholesteatoma in a Japanese population

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Authors: Satoshi Hara M.D. [a,b]*, Takeshi Kusunoki M.D., Ph.D. [b], Hiroshi Nakagawa Ph.D. [c], Yu Toyoda Ph.D. [d], Shuko Nojiri Ph.D. [e], Kazusaku Kamiya Ph.D. [b], Masayuki Furukawa M.D., Ph.D. [b], Yusuke Takata M.D., Ph.D. [b], Hiroko Okada M.D., Ph.D. [b], Takashi Anzai M.D., Ph.D. [b], Fumihiko Matsumoto M.D., Ph.D. [b], Katsuhisa Ikeda M.D., Ph.D. [b]

[a]Department of Otorhinolaryngology, Juntendo University Graduate School of Medicine, Tokyo, Japan

[b]Department of Otorhinolaryngology, Juntendo University Faculty of Medicine, Tokyo, Japan

[c]Department of Applied Biological Chemistry, Graduate School of Bioscience and Biotechnology, Chubu University, Japan

[d]Department of Pharmacy, The University of Tokyo Hospital, Tokyo, Japan

[e]Medical Technology Innovation Center, Juntendo University, Japan

***Corresponding author:** Satoshi Hara, Department of Otorhinolaryngology, Juntendo University Faculty of Medicine, 1-5-29, Yushima, Bunkyo-ku, Tokyo 113-0034, Japan, Tel: +81358021229, Fax: +81358407013, E-mail: s-hara@juntendo.ac.jp, ORCID: 0000-0002-5199-8363

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Keywords. *ABCC11*, acquired middle ear cholesteatoma, earwax, apocrine gland, volatile organic compounds

Abstract

Objective. A single nucleotide polymorphism 538G>A in the human *ABCC11* gene is a determinant of the earwax morphotype. *ABCC11* 538GG and GA correspond to wet earwax and 538AA to dry earwax. Despite a putative positive correlation between the frequency of the 538G allele and prevalence of cholesteatoma, minimal clinical information is currently available. We aimed to evaluate this association between the *ABCC11* genotypes and acquired middle ear cholesteatoma.

Study Design. Case-control study.

Setting. Single center, academic hospital.

Methods. We recruited 67 Japanese patients with acquired middle ear cholesteatoma (cholesteatoma group) and 100 Japanese controls with no history of middle ear cholesteatoma. We assessed the *ABCC11* genotypes for all participants. Clinical information was collected from the cholesteatoma group. The genotype data of 104 Japanese people from the 1000 Genomes Project that represent the general population were used.

Results. The proportion of participants with *ABCC11* 538GG or GA was significantly higher in the cholesteatoma group than that in the control group or general Japanese population ($p <$ 0.001). The *ABCC11* 538G allele frequency was also significantly higher in the

cholesteatoma group than that in the control group or general Japanese population ($p <$ 0.001). Multivariate logistic regression analyses revealed a significant association between the *ABCC11* genotype and acquired middle ear cholesteatoma (odds ratio = 5.49; 95% confidence interval = 2.61–11.5; $p < 0.001$).

Conclusion. Our results suggest that the *ABCC11* genotypes could be associated with the development of acquired middle ear cholesteatoma among Japanese people.

Introduction

Middle ear cholesteatoma is a destructive nonneoplastic lesion of the temporal bone that can gradually expand and cause complications by bone erosion[1]. The pathogenesis of middle ear cholesteatoma remains controversial. It has been proposed that the external features in the external auditory canal (EAC) such as earwax and otitis externa may contribute to the development of acquired middle ear cholesteatoma[2,3]; however, there have been few studies demonstrating this association.

The morphotypes of earwax are determined by a non-synonymous single nucleotide polymorphism 538G>A (rs17822931: Gly180Arg) in the human *ATP-binding cassette transporter C11* (*ABCC11*) gene as follows: *ABCC11* 538GG and GA are wet earwax genotypes, and 538AA is a dry earwax genotype[4]. The *ABCC11* gene encodes an ATP-driven efflux pump protein found in the EAC's apocrine glands, which produce earwax along with the sebaceous glands[4-6]. Biochemical analyses have demonstrated that the Gly180Arg variant is functionally null, since the protein undergoes proteasomal degradation[7]. Actually, ABCC11 protein was not detected in human apocrine glands carrying *ABCC11* 538AA[8]; such apocrine glands were not reportedly well-developed, which might be associated with loss of histological function[9]. Besides, a previous study has shown that the *ABCC11* 538G allele is

dominant, while the 538A allele is a recessive (null) allele[4]. It has been previously reported that the *ABCC11* 538G allele is dominant in American, African, and European populations whereas the *ABCC11* 538A allele is dominant in East Asian populations[4]. Interestingly, the incidence of cholesteatoma in the United states (6.0/100000 per year[10]) and European countries (6.8–15.5/100000 per year[11-13]) is reportedly higher than that in East Asia (3.9/100000 per year[14]). This trend seems similar to the observed ethnic differences in the frequency of *ABCC11* 538G>A[15]; the 538G allele frequencies in Americans (0.860), Europeans (0.864), and Africans (0.988) are higher than that in East Asians (0.220), based on the most recent phase 3 data from the 1000 Genomes Project[16]. Given this association, *ABCC11* 538G>A may be related to acquired middle ear cholesteatoma[17]; however, this association has not been extensively explored.

The objective of our observational study was to assess the association between the *ABCC11* genotypes at site 538 and acquired middle ear cholesteatoma among affected patients, compared with control participants and the general Japanese population.

Material and Methods

Participants

This study was approved by the Juntendo University Committee for Ethics concerned with the human genome (identification number 2020002). This study was conducted in accordance with the principles for human experimentation, as defined in the 1964 Declaration of Helsinki and its later amendments.

Between April 2013 and March 2020, 77 patients were diagnosed with middle ear cholesteatoma at our hospital, underwent a surgical procedure, and provided written consent to participate in this study. The inclusion criteria consisted of being aged 15 years or older and being Japanese. Patients who were diagnosed with congenital middle ear cholesteatoma were excluded from this study. In total, we excluded 10 patients because 4 were below 15 years of age, and 6 were diagnosed with congenital middle ear cholesteatoma. Finally, 67 patients were enrolled (cholesteatoma group).

During the study period, 100 individuals with no history of cholesteatoma who were admitted to our hospital for various surgical procedures with blood tests comprised the control group. Each participant's sex and age were recorded. Blood samples were collected from all participants.

In order to generally include healthy participants, we compared our data with the genotype distribution of *ABCC11* in 104 Japanese individuals in Tokyo, Japan, which was the

most recent data from the 1000 Genomes Project (phase 3)[16]. These data showed that the number of individuals carrying *ABCC11* 538GG, GA, and AA genotypes were 2, 21, and 81, respectively. In this population, the proportion of the individuals carrying the wet earwax genotypes was 22%, and the *ABCC11* 538G allele frequency was 0.120.

Because the *ABCC11* 538G allele is dominant and 538A allele is the null allele[4], the genotypes were compared in a dominant model in this study.

Characterization of the Cholesteatoma group

In the cholesteatoma group, we evaluated the (i) affected side of the ear; (ii) proportion of bilateral cholesteatoma; (iii) proportion of recurrence of cholesteatoma; (iv) preoperative air-conduction hearing level; (v) preoperative air-bone gap; (vi) history of recurrent acute otitis media; (vii) history of otitis media with effusion; (viii) patulous eustachian tubes; (ix) tympanic membrane perforation; (x) preoperative stage of the cholesteatoma; and (xi) ossicular destruction.

The preoperative air- and bone-conduction hearing levels were calculated as the means of the thresholds obtained at 500, 1000, and 2000 Hz. The air-bone gap was reported as the difference between the air- and bone-conduction values that were determined simultaneously. Hearing results were determined at the last follow-up. For each patient, each ossicle's status

was evaluated intraoperatively according to the rating criteria, as previously described (Table 1)[18]. Cholesteatoma staging was done according to the European Academy of Otology and Neurotology/the Japanese Otological Society staging system (Table 2)[19]. The patients with acquired middle ear cholesteatoma were divided into two groups: 1) patients carrying the wet earwax genotypes, and 2) patients carrying the dry earwax genotype. To confirm that the earwax morphotypes matched the *ABCC11* genotype, the morphotypes of earwax from ten individuals in the two groups were examined using a microscope. All of them were found to match.

Genotyping

The genotypes of *ABCC11* 538G>A were examined as previously described[7,20]. Briefly, all blood samples were collected in standard 2Na-EDTA-coated blood collection tubes. The samples were subjected to proteinase K digestion; genomic DNA was then isolated using phenol/chloroform extraction. Before sequencing, a part of the *ABCC11* gene, including the 538G>A allele, was amplified from the genomic DNA by polymerase chain reaction (PCR) with the following primer set: forward 5′-aacaaagctcctggctagcaag-3′, and reverse 5′-ccataaggtctacacctgagggtc-3′. The amplicons were then subjected to ExoSAP-IT (Cytiva, Tokyo, Japan) treatment. Samples for sequencing were then prepared using a specific primer

(5′-tcctggctagcaagaactaggatg-3′ or 5′-attccatggggaaaccaagtc-3′) and BigDye Terminator 3.1 (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. The sequence information obtained with the Autosequencer Model 3100 (Applied Biosystems) was aligned with an AutoAssembler (Applied Biosystems) and visualized using Sequencher 4.7 Demo (Hitachi Software Engineering, Tokyo, Japan). All genotyping procedures were conducted by an individual blinded to the patients' information and clinical data.

Statistics

Descriptive statistics were presented as medians (interquartile ranges [IQRs]) for the continuous variables. The Mann–Whitney U test was used to compare the continuous variables. χ^2 and Fisher's exact tests were used to compare the proportions of categorical variables. The Hardy-Weinberg equilibrium (HWE) of the genotype frequency distribution for *ABCC11* was tested in all groups using Fisher's exact tests to confirm that the allele counts were statistically sufficient.

Univariate and multivariate logistic regression analyses were performed to estimate the association between the *ABCC11* genotype and acquired middle ear cholesteatoma. For these

logistic regression analyses, we included variables to adjust for confounding factors, such as sex and age. A p value less than 0.05 was considered statistically significant. All analyses were performed using SPSS Statistics version 26 (IBM, Armonk, NY, USA).

Results

Profiles of the participants in the cholesteatoma group, the control group, and the general Japanese population are summarized in Table 3. There was no significant difference in sex between the cholesteatoma group and the control group; however, the age was significantly higher in the cholesteatoma group than in the control group ($p = 0.018$). The genotype frequency distribution of *ABCC11* was in HWE in all groups. The proportion of participants carrying the wet earwax genotypes (538GG and GA) was significantly higher in the cholesteatoma group than in the control group ($p < 0.001$) and in the general Japanese population ($p < 0.001$). Notably, there was no significant difference ($p = 0.27$) between the proportions of individuals carrying the wet earwax genotypes in the control group and the general Japanese population, suggesting an accurate sample collection in this study (Figure 1). *ABCC11* 538G allele frequency was also significantly higher in the cholesteatoma group than in the control group ($p < 0.001$) and in the general Japanese population ($p < 0.001$).

Next, to investigate the association of the *ABCC11* genotypes with severity of acquired middle ear cholesteatoma and history of possible predisposition to the disease, we compared the profiles of patients with wet earwax genotypes to those with the dry earwax genotype in the cholesteatoma group (Table 4). The proportion of recurrence of cholesteatoma was 33% (11/33 patients) in the patients carrying the wet earwax genotypes and 21% (7/34 patients) in the patients carrying the dry earwax genotype. Although the proportion was higher in the former group, the difference was not statistically significant ($p = 0.24$). The proportion of the history of recurrent acute otitis media in the patients carrying the wet earwax genotypes (9%) was lower than that in the patients carrying the dry earwax genotype (27%), although the difference was not statistically significant ($p = 0.064$). There were no significant differences in the other parameters between the cholesteatoma patients with wet and dry earwax genotypes.

Finally, we performed univariate and multivariate logistic regression analyses for potential predictors of acquired middle ear cholesteatoma in the cholesteatoma group and the control group (Table 5). Univariate logistic regression analyses revealed that acquired middle ear cholesteatoma was associated with age (odds ratio [OR] = 1.02; 95% confidence interval [CI] = 1.00–1.04; $p = 0.023$) and with the wet earwax genotypes (OR = 5.10; 95% CI = 2.49–

194 10.4; $p < 0.001$). Multivariate logistic regression analysis showed that age was significantly
195 associated with acquired middle ear cholesteatoma (OR = 1.03; 95% CI = 1.01–1.05; $p <$
196 0.015). Moreover, the wet earwax genotypes were significantly associated with acquired
197 middle ear cholesteatoma after adjusting for confounding factors (OR = 5.49; 95% CI =
198 2.61–11.5; $p < 0.001$). Although the age in the control group was significantly higher than
199 that in the cholesteatoma group, the *ABCC11* genotype was found to be significantly
200 associated with the development of acquired middle ear cholesteatoma after adjusting for
201 confounding factors including age. Regarding the wet earwax genotypes, the OR in
202 univariate logistic regression analysis (5.10) was different from that in multivariate logistic
203 regression analysis (5.49), supposedly due to the confounding effects of age and sex.
204
205 **Discussion**

206 *The association between the ABCC11 genotype at site 538 and the development of acquired*
207 *middle ear cholesteatoma*

208 To the best of our knowledge, this study investigated, for the first time, the association
209 between the *ABCC11* genotypes at site 538 and acquired middle ear cholesteatoma by
210 comparing patients with the disease, control participants, and the general Japanese

211 population. The proportion of individuals carrying the wet earwax genotypes was
212 significantly higher in the cholesteatoma group than in the control group and the general
213 Japanese population (Table 3, Figure 1). Multivariate logistic regression analyses revealed
214 that the *ABCC11* genotype was significantly associated with the development of acquired
215 middle ear cholesteatoma (Table 5).
216 When comparing the profiles of patients with the wet earwax genotypes to those with
217 the dry earwax genotype in the cholesteatoma group (Table 4), the proportion of recurrence
218 of cholesteatoma tended to be higher in patients carrying the wet earwax genotypes. On the
219 other hand, the proportion of the history of recurrent acute otitis media in the patients
220 carrying the wet earwax genotypes tended to be lower than that in the patients carrying the
221 dry earwax genotype. Nevertheless, further studies are needed to validate these findings.
222 Interestingly, the preoperative severity of the disease, indicated by the air conduction hearing
223 level, air-bone gap, staging, and degree of ossicular destruction, did not differ between
224 patients with the disease carrying the wet earwax genotypes and patients carrying the dry
225 earwax genotypes. Our previous study reported that Japanese patients with wet earwax
226 genotypes tended to develop bilateral rather than unilateral middle ear cholesteatoma[20].
227 However, in this study, the proportion of bilateral cholesteatoma did not differ between

patients carrying the wet and dry earwax genotypes. To clarify this point, further studies are required.

Hypothesis of the mechanism underlying the association between the ABCC11 genotypes at site 538 and acquired middle ear cholesteatoma

In the EAC, it has been reported that the *ABCC11* genotype is associated with the earwax morphotype, the development of apocrine glands, and the composition of volatile organic compounds (VOCs)[4,7,21].

Regarding the earwax morphotype, wet earwax is sticky, whereas dry earwax lacks cerumen[4]. It has been proposed that the blockage of debris transport by earwax can cause acquired middle ear cholesteatoma[2]. In this context, the different tendencies of wet and dry morphotypes to accumulate earwax may be the mechanism underlying the association between the *ABCC11* genotypes and acquired middle ear cholesteatoma.

Regarding the association between the *ABCC11* genotype and apocrine glands, well-developed apocrine glands have been observed in the EACs of patients carrying the wet earwax genotypes as opposed to the dry earwax genotype[7,8]. Furthermore, the proportion of subjects with wet earwax is higher in patients with hidradenitis suppurativa, which is an inflammatory skin disease in the apocrine gland-bearing skin, than that in the general

245 Japanese population[22,23]. It has been proposed that otitis externa with high epithelial turnover
246 can contribute to acquired middle ear cholesteatoma[2,3]; therefore, the maintenance of
247 apocrine glands may be associated with this disease.

248 It has been proposed that VOCs in earwax are produced from bacterial modification of
249 earwax-based compounds in the EAC[21]. Additionally, the amounts of several kinds of VOCs
250 are greater in wet earwax than in dry earwax[21]. Exposure to VOCs can lead to skin
251 inflammation, likely because of their toxicity due to oxidative stress[24,25]. Therefore, the high
252 amounts of VOCs in wet earwax genotypes may be associated with the development of
253 acquired middle ear cholesteatoma via otitis externa.

254 The hypothesis that the wet earwax genotypes are associated with the development of
255 otitis externa is supported by previous studies[26-28]. The bactericidal activities of dry earwax
256 are reported to be no less than those of the wet one and to even exceed those of the wet one
257 for some bacteria[26,27]. Furthermore, higher lysozyme activities and increased levels of
258 immunoglobulins, which are known to play a role in resistance to infection, have been found
259 in dry earwax than in wet earwax[28]. Therefore, it is plausible that the wet earwax genotypes
260 are associated with the development of otitis externa.

We hypothesized some mechanisms of the association between the *ABCC11* genotypes and acquired middle ear cholesteatoma. However, the underlying mechanism of the association between the *ABCC11* genotypes and cholesteatoma is still unknown. Therefore, further studies are needed to elucidate the pathogenesis of cholesteatoma.

Additionally, based on our hypothesis, the *ABCC11* genotypes may also be associated with diseases attributed to otitis externa, such as keratosis obturans and external auditory canal cholesteatoma[29,30]. However, further studies are required to investigate this aspect.

Contribution of this study

Our results can contribute to the prediction of the potential risk of acquired middle ear cholesteatoma; they suggest an increased risk of the disease in individuals carrying the wet earwax genotypes and a decreased risk of the disease in individuals carrying the dry earwax genotype. The *ABCC11* genotypes (538GG/GA or AA) can be identified by clinically checking the earwax phenotype. Therefore, the potential risk of acquired middle ear cholesteatoma may be easily predicted without genetic testing. Checking the earwax phenotype would be an easy and low-cost method for the prediction of the potential risk of acquired middle ear cholesteatoma. This approach may be particularly useful in East Asian countries, because the proportions of wet and dry earwax genotypes are less polarized among

East Asian populations; 37% wet earwax and 63% dry earwax genotypes are seen among East Asian populations, whereas more than 98% wet earwax genotypes are found among American, European, and African populations, based on the most recent data from the 1000 Genomes Project (phase 3)[16]. Furthermore, this low-cost approach will be feasible for low-income and middle-income countries.

Our results suggest that the investigation of the association between the *ABCC11* genotypes and acquired middle ear cholesteatoma could contribute to the prevention of its development. Changing the EAC environment of individuals with wet earwax genotypes to more closely resemble that of individuals carrying the dry earwax genotype may decrease the risk for acquired middle ear cholesteatoma. Although the underlying mechanism of the association between the *ABCC11* genotypes and cholesteatoma is still unknown, the prevention of acquired middle ear cholesteatoma would be particularly helpful for countries with a high incidence of cholesteatoma and a high ratio of wet earwax genotypes, such as American, European, and African countries.

In this context, our findings may contribute to elucidating the pathogenesis of cholesteatoma and developing new diagnostic and therapeutic strategies.

Limitation of this study

This study is potentially limited by its case-control design and the small number of individuals who were Japanese and referred for surgery in central Tokyo. Because the genotype frequency distribution of *ABCC11* varies by ethnic population[4], further studies across different countries and ethnic populations are needed. In this study, the *ABCC11* genotypes at site 538 were compared among the subjects only in a dominant model, given the small number of patients with the *ABCC11* 538GG genotype and the fact that 538A is a null allele[4]. Nonetheless, our results suggest that the *ABCC11* genotypes are associated with the development of acquired middle ear cholesteatoma.

Conclusion

To our knowledge, this is the first report of the investigation of the *ABCC11* genotypes at site 538 in patients with acquired middle ear cholesteatoma, control participants, and the general Japanese population. Our case-control study showed that the *ABCC11* genotypes at site 538 was associated with the acquired middle ear cholesteatoma among Japanese people. Further studies are needed to reveal the molecular mechanisms of this association.

311

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316

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390

391

392 **Table 1.** Criteria for rating the status of ossicles

Rating	Criteria
1	Completely normal
2	Cholesteatoma abuts the ossicle, but the ossicle is still intact
3	The ossicle is partially eroded by cholesteatoma
4	The ossicle is completely absent (for the malleus and incus) or if the superstructure is eroded (for the stapes).

The status of each ossicle of patients with cholesteatoma was evaluated intraoperatively, according to the criteria for rating as previously described[18].

393

394 **Table 2.** Staging system for cholesteatoma

Staging	Criteria
I	Cholesteatoma localized in the primary site
II	Cholesteatoma involving two or more sites
III	Cholesteatoma with extracranial complications
IV	Cholesteatoma with intracranial complications

Cholesteatoma staging of patients with cholesteatoma was done according to the European Academy of Otology and Neurotology/the Japanese Otological Society staging system[19].

395

Table 3. Profiles of the participants in the cholesteatoma group, control group, and general Japanese population

Characteristics	Cholesteatoma group (*n* = 67)	Control group (*n* = 100)	*P*	General Japanese population (*n* = 104)	*P*
Sex (male), *n* (%)†	37 (55%)	55 (55%)	0.98		
Age (years), median (IQRs)	63.0 (44.0, 72.0)	50.0 (35.0, 65.0)	0.018*		
Genotypes of the *ABCC11*, *n* (%)‡			<0.001** *		<0.001** *
538GG	2 (3.0%)	1 (1.0%)		2 (1.9%)	
538GA	31 (46%)	15 (15%)		21 (20%)	
538AA	34 (51%)	84 (84%)		81 (78%)	
ABCC11 538G allele frequency	0.261	0.085	<0.001** *	0.120	<0.001** *

The *ABCC11* genotype data of 104 Japanese individuals in Tokyo, Japan, from the 1000 Genomes Project (phase 3) were used to represent the general Japanese population[16]. The *P* value was calculated by comparing cholesteatoma group with control group and cholesteatoma group with the general Japanese population.

IQR, interquartile range; *ABCC11*, *adenosine triphosphate-binding cassette transporter C11*; †, χ^2 test; ‡, Fisher's exact test; *, $p < 0.05$; ***, $p < 0.001$

398

399 **Table 4.** Profiles of the patients carrying the wet and the dry earwax genotypes in the

400 cholesteatoma group

Characteristics	Patients carrying the wet earwax genotypes (*n* = 33)	Patients carrying the dry earwax genotype (*n* = 34)	*P*
Sex (male), *n* (%)†	18 (55%)	19 (56%)	0.91
Age (years), median (IQRs)	64.0 (38.0, 73.0)	63.0 (38.0, 71.3)	0.39
Affected side (right), *n* (%)†	15 (45%)	18 (53%)	0.54
Proportion of bilateral cholesteatoma, *n* (%)†	14 (42%)	14 (41%)	0.92
Proportion of recurrence of cholesteatoma, *n* (%)†	11 (33%)	7 (21%)	0.24
Preoperative air conduction hearing level (dB), median (IQRs)	50.0 (34.2, 70.9)	51.7 (30.9, 73.4)	0.90
Preoperative air-bone gap	30.0 (23.3, 42.5)	27.5 (19.6, 42.1)	0.54

(dB), median (IQRs)			
History of recurrent acute otitis media, *n* (%)†	3 (9%)	9 (27%)	0.064
History of otitis media with effusion, *n* (%)‡	3 (9%)	3 (9%)	1.00
Patulous eustachian tubes, *n* (%)‡	6 (18%)	4 (12%)	0.51
Tympanic membrane perforation, *n* (%)‡	2 (6.1%)	3 (8.8%)	1.00
Preoperative stage, *n* (%)‡			1.00
I	3 (9.1%)	5 (15%)	
II	23 (70%)	18 (53%)	
III	7 (21%)	11 (32%)	
IV	0 (0%)	0 (0%)	
The rate of ossicular destruction, *n* (%)‡			0.47
1	1 (3%)	1 (3%)	

2	9 (27%)	9 (26%)
3	12 (36%)	13 (34%)
4	11 (33%)	11 (32%)

Wet earwax genotypes, *ABCC11* 538GG and GA genotypes; Dry earwax genotype, *ABCC11* 538AA genotype; *ABCC11*, *adenosine triphosphate-binding cassette transporter C11*; IQR, interquartile range; †, χ^2 test; ‡, Fisher's exact test

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403 **Table 5.** Univariate and multivariate logistic regression analyses for the association between

404 the *ABCC11* genotype and acquired middle ear cholesteatoma in the cholesteatoma group and

405 the control group

Characteristics	Univariate		Multivariate	
	OR (95% CI)	*P*	OR (95% CI)	*P*
Age	1.02 (1.00–1.04)	0.023*	1.03 (1.01–1.05)	0.015*
Sex	0.99 (0.53–1.85)	0.98	0.78 (0.39–1.56)	0.49
Wet earwax genotypes (vs. dry earwax genotype)	5.10 (2.49–10.4)	<0.001***	5.49 (2.61–11.5)	<0.001***

OR, Odds ratio; CI, confidence interval; Wet earwax genotypes, *ABCC11* 538GG and GA genotypes; Dry earwax genotype, *ABCC11* 538AA genotype; *ABCC11, adenosine triphosphate-binding cassette transporter C11*; *, $p < 0.05$; ***, $p < 0.001$

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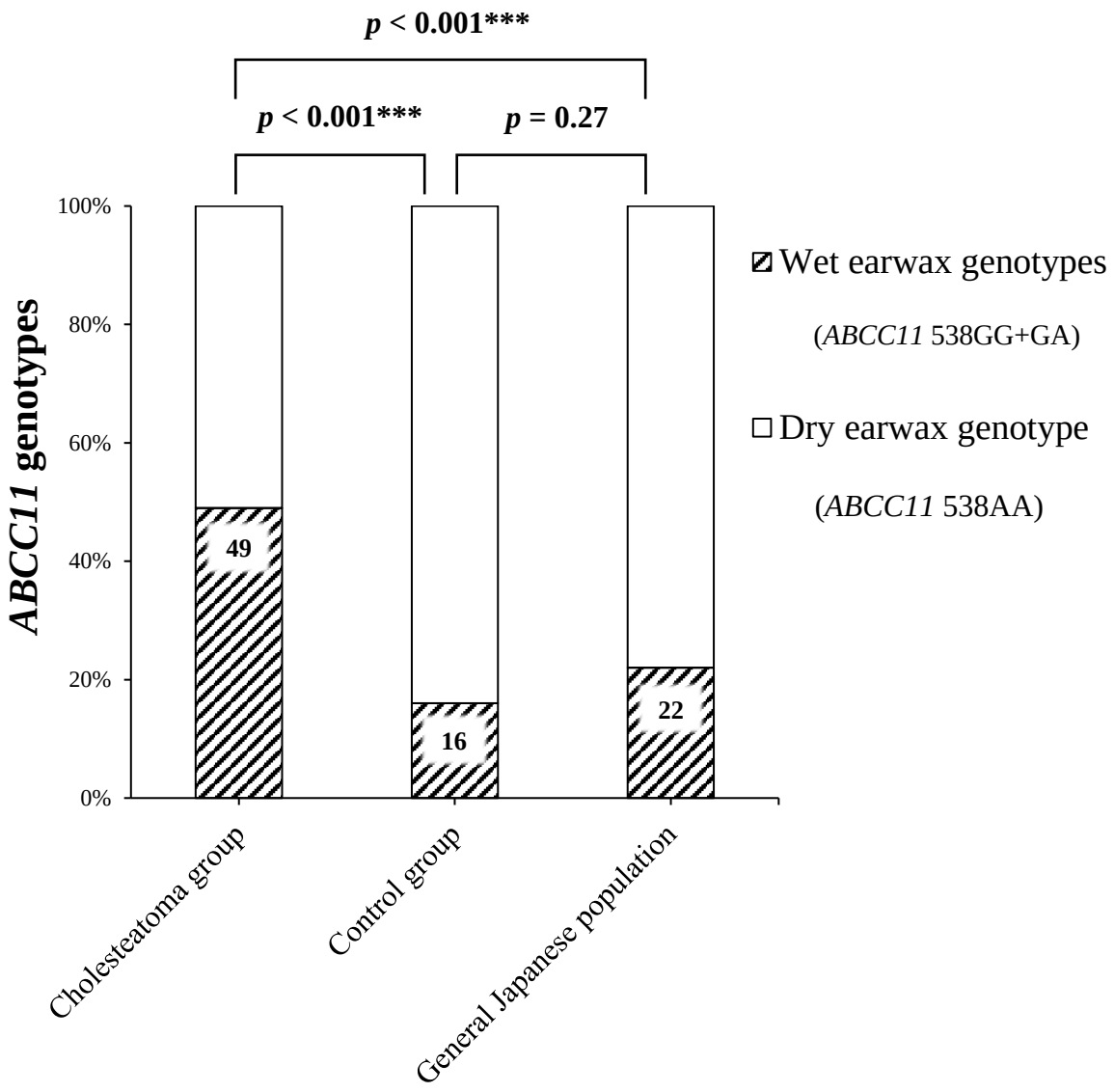
407

Figure legend

Figure 1. *ABCC11* genotypes in the cholesteatoma group, control group, and general Japanese population

***, $p < 0.001$


p < 0.001***
p < 0.001***
p = 0.27
100%
80%
60%
40%
20%
0%
ABCC11 genotypes
49
16
22
Wet earwax genotypes
(ABCC11 538GG+GA)
Dry earwax genotype
(ABCC11 538AA)
Cholesteatoma group
Control group
General Japanese population