the *ABCC12* gene in mammals (10, 11), but no gene orthologous to human *ABCC11* (11) has been found in mammals except for primates. Based on a database search, it is speculated that *ABCC11* is a paralogous gene generated by *ABCC12* gene duplication in the primate genome, including that of humans (12). Human ABCC11 reportedly functions as an ATP-dependent efflux pump for amphipathic anions, including cyclic nucleotides, leukotriene C_4, estrone 3-sulfate, dehydroepiandrosterone 3-sulfate (DHEAS), estradiol 17-β-D-glucuronide, and folic acid (13-16).

We have recently reported that one single-nucleotide polymorphism (SNP), 538G>A (Gly180Arg), in the *ABCC11* gene determines the type of earwax (6). This is the first example of a DNA polymorphism determining a visible genetic trait. The G/G and G/A genotypes correspond to the wet type of earwax, whereas A/A corresponds to the dry type. A functional assay has demonstrated that cells with allele A show a lower excretory activity for cGMP than those with allele G (6). Wide ethnic differences were also observed in the frequencies of those alleles (6). Furthermore, it has most recently been demonstrated that the genotype frequencies in the *ABCC11* and *EDAR* genes differ significantly between the Hondo and Ryukyu clusters in Japan (17).

At the present time, however, little is known about how genetic polymorphisms of the *ABCC11* gene affect the function of the ceruminous apocrine gland. The present study has two specific aims. First, to understand the molecular mechanism determining the earwax type, we investigated the effect of the SNP 538G>A (Gly180Arg) on the protein expression and intracellular localization of ABCC11. Second, to gain insight into the link between the genetic polymorphisms of *ABCC11* and the functions of apocrine glands, such as cerumen secretion and axillary osmidrosis, we designed a primer set for clinical genotyping.

In this article, we provide evidence that the SNP 538G>A (Gly180Arg) variant of human ABCC11 lacking *N*-linked glycosylation is recognized as a misfolded protein in the endoplasmic reticulum (ER) and readily undergoes proteasomal degradation. This ABCC11 protein degradation underlies the molecular mechanism to form the dry type of earwax as a mendelian trait with a recessive phenotype. Furthermore, we demonstrate that the wet type of earwax is genetically linked with axillary osmidrosis, which is recognized as a disease covered by the national health insurance system in Japan. Human ABCC11 is suggested to play a pivotal role in the secretion of steroid metabolites from secretory cells in the apocrine glands.

MATERIALS AND METHODS

Collection of genomic DNA from volunteers and preparation of DNA samples

Under written informed consent, we collected blood samples from 124 Japanese volunteers at Nagasaki University (Nagasaki, Japan) during a period from January 2004 to December 2005. Genomic DNA was extracted from whole blood by the standard method. Protocols for the present study were approved by the Committee for the Ethical Issues on Human Genome and Gene Analysis, Nagasaki University. The clinical investigation was conducted according to the Declaration of Helsinki Principles. Briefly, all blood samples were collected in standard 2Na-EDTA-coated blood collection tubes. The samples were subjected to proteinase K digestion, and then genomic DNA was isolated by phenol/chloroform extraction and subsequently by ethanol precipitation. Part of the *ABCC11* gene including the 538G>A alleles was amplified by PCR with the following primers: EWX1, 5′-TGCAAAGAGATTCCACCAGTT-3′; and EWX2, 5′-AAGGTCTTCATTTTCTAGACAGC-3′.

Diagnosis of axillary osmidrosis in Japanese patients and genotyping

All procedures for the diagnosis of axillary osmidrosis patients and their genotyping were performed according to the protocol approved by the Ethical Review Board of Fujita Health University School of Medicine (Nagoya, Japan). The clinical investigation was conducted according to the Declaration of Helsinki Principles. Fourteen axillary osmidrosis patients participated in this study under written informed consent. All of the patients expressed their subjective views on symptoms by answering survey inquiries. Axillary osmidrosis was diagnosed basically from both the self-declaration of patients and the odor-smelling test carried out by authorized medical doctors. Earwax type was determined from wax samples obtained by stirring a cotton swab in the external auditory canal of the patient. Individual genomic DNA was obtained from 2 ml of whole blood by using QuickGene-610L (Fujifilm Co., Tokyo, Japan) according to the manufacturer's protocol. To sequence the SNP 538G>A, the following PCR primers were designed: P1, 5′-TGTCACATGCAAAGAGATTCC-3′; and P2, 5′-CTCCTGGCATGGACTTGAACA-3′. To identify the Δ27 mutation, we designed one set of primers: P3, 5′-AGGTCTCTAGGGCCTGAAGTA-3′; and P4, 5′-AGCCTTCACCTTCCCATTGCC-3′. The experiments to determine the genotype of each patient with *ABCC11* gene amplification by PCR and DNA sequencing were carried out in a masked manner.

DNA sequence analysis

DNA sequence analysis was carried out by using BigDye Terminator 3.1 (Applied Biosystems Inc., Foster City, CA, USA) after ExoSAP-IT treatment, and with an Autosequencer Model 3100 (Applied Biosystems Inc.), according to the manufacturer's protocol. The sequences were aligned with an AutoAssembler (Applied Biosystems Inc.) and visualized with Sequencher 4.7 Demo (Hitachi Software Engineering Co., Ltd., Tokyo, Japan) to find SNPs or mutations.

Data analysis

SNP data on the polymorphisms of ABCC11 were obtained from the dbSNP database of the National Center for Biotechnology Information (NCBI; Bethesda, MD, USA). The hydropathy profile of the ABCC11 protein deduced from the cDNA sequence was calculated with the Kyte-Doolittle hydropathy algorithm (18). Potential *N*-linked glycosylation sites were predicted with Genetyx-Win 5.1. (Software Development Co., Ltd., Tokyo, Japan)

Polyclonal antibody against ABCC11

The cytoplasmic domain (aa 746–804) close to the first ATP-binding cassette, located between transmembrane domains 6 and 7 (TM6 and TM7) of the ABCC11 protein, was selected as an epitope for development of the polyclonal antibody. The epitope-encoding cDNA (2236-2412) was inserted into the pGEX-2T vector (GE Healthcare, Little Chalfont, UK), and then *Escherichia coli* strain BL21 [F^-, *ompT*, *hsdS*(r_B^-, m_B^-), gal] cells were transformed with the vector to produce a glutathione-*S*-transferase (GST)-like fusion peptide. The expressed fusion peptide was isolated by using a glutathione-affinity gel column (Pierce, Rockford, IL, USA) according to the manufacturer's protocol. The epitope peptide was then recovered by PreScission Protease digestion (GE Healthcare). The anti-ABCC11 polyclonal antibody was produced by immunization of rabbits with the epitope peptide. The antiserum was purified through an affinity column and used for immunohistochemistry and immunoblotting studies.

Immunohistochemical detection of ABCC11 expressed in the ceruminous apocrine gland

External auditory canal tissues were obtained, with informed consent, from a patient undergoing surgical excision of a squamous cell carcinoma around the ear at Nagasaki University. The tissue samples were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) at 4°C overnight, embedded in paraffin, and sectioned at 4 μm in thickness. After antigen retrieval by microwave treatment in 0.01 M citrate buffer (pH 6.0), deparaffinized sections were preincubated with 10% normal goat serum. Thereafter, tissues were reacted with anti-ABCC11 rabbit polyclonal antibody at 1:500 dilution. The slides were subsequently incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody (Invitrogen Co., Carlsbad, CA, USA). Specimens were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI-I; Vysis Inc., Downers Grove, IL, USA), visualized, and photographed with a fluorescence microscope (Zeiss Axioplan2; Carl Zeiss Japan, Tokyo, Japan) equipped with a charge-coupled device camera (19).

Expression of ABCC11 in Sf9 insect cells

The cDNA of human ABCC11 WT was inserted into the pFastBac1 vector (Invitrogen) between the restriction enzyme sites of *Xba*I and *Hind*III. To express ABCC11 in insect cells, recombinant baculoviruses were generated with the Bac-to-Bac baculovirus expression systems (Invitrogen) according to the manufacturer's protocols. Insect *Spodoptera frugiperda* Sf9 cells (1.0×10^6 cells/ml) were infected with the recombinant baculovirus and cultured in NIM-Ex Insect serum-free medium (Nosan Co., Yokohama, Japan) supplemented with penicillin (100 U/ml), and streptomycin (100 μg/ml) (Invitrogen) with gentle shaking at 28°C. Three days after infection, cells were harvested by centrifugation. To prepare the whole-cell lysate, cells were subsequently washed with PBS and collected by centrifugation.

Generation of ABCC11 variant forms

The human ABCC11 WT or G180R cDNA was inserted into the pcDNA3.1/Hygro(−) vector (Invitrogen) between the restriction enzyme sites of *Xho*I/*Sal*I and *Hind*III, respectively. The resulting expression construct [ABCC11 WT-pcDNA3.1/Hygro(−)] was used as the template for site-directed mutagenesis to obtain ABCC11 variants, *i.e.*, N838Q, N844Q, and N838Q/N844Q. To generate Asn-to-Gln variant forms of ABCC11, a codon (AAT) encoding asparagine was converted to CAA by using *Pfu Turbo* DNA polymerase and the QuikChange site-directed mutagensis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol. The sequences of internal complementary PCR primers used for the site-directed mutagenesis, as well as the corresponding PCR conditions, are summarized in Supplemental Table 1. To generate the N838Q/N844Q variant, ABCC11 N838Q-pcDNA3.1/Hygro(−) was used as the template. Each variant cDNA generated in the pcDNA3.1/Hyg(−) plasmid was subjected to nucleotide sequence analysis (Shimadzu Co., Kyoto, Japan). To substitute Gly180 to Arg, Lys, His, Asp, Glu, AL, or Pro in the ABCC11 WT protein, the codon (GGG) encoding the Gly residue in TM1 was changed by site-directed mutagenesis, as described above. The PCR primers used for the site-directed mutagenesis are shown in Supplemental Table 2. Furthermore, hitherto known nonsynonymous SNP variants of ABCC11 were generated in the same manner as described above (see Supplemental Table 3 for the PCR primers used for site-directed mutagenesis).

Expression of ABCC11 WT and variants in Flp-In-293 cells

Flp-In-293 cells (Invitrogen) were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified atmosphere at 37°C with 5% CO_2 in air, as described previously (20-22). Flp-In-293 cells were replated onto 35-mm dishes at a concentration of 1.0×10^6 cells/dish. After 24 h, the cells were transfected with the ABCC11-pcDNA3.1/Hyg(−) vector by using Lipofectamine 2000 (Invitrogen). The amount of plasmid DNA used for transfection was adjusted to be the same among ABCC11 WT and its variants. To inhibit proteasomal degradation when needed, cells at 24 h after transfection were cultivated in the presence of 2 μM MG132 for a further 24 h.

At 48 h after the transfection, cells were washed with PBS and then treated with lysis buffer A containing 50 mM Tris/HCl (pH 7.4), 1% (w/v) Triton X-100, 1 mM dithiothreitol, and protease inhibitor mixture (CompleteTM Mini; Nacalai Tesque, Inc., Kyoto, Japan). The cell suspension sample was homogenized by passage through a 27-gauge needle and then centrifuged at 800 *g* at 4°C for 10 min. The resulting supernatant fraction (whole-cell lysate) was transferred to a new tube, and the protein concentration was quantified by using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

Detection of ABCC11 mRNA by reverse transcription-PCR

Total RNA was extracted from the ABCC11-transfected cells with the High-Pure RNA Isolation Kit (Roche Ltd., Mannheim, Germany) according to the manufacturer's protocol. cDNA was prepared from the extracted RNA in a reverse transcriptase reaction by using the High Capacity cDNA Archive kit (Applied Biosystems Inc.) and random hexamers, according to the manufacturer's instructions. The mRNA levels of ABCC11 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined by PCR in an iCycler™ thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with the following specific primers: ABCC11, 5′-AGCTTACGGAGTCGCTTAGATGA-3′ and 5′-TGGCTGTGCGTTGGTTGA-3′; and GAPDH, 5′-AATTCCATGGCACCGTCAA-3′ and 5′-CATGAGTCCTTCCACGATACCA-3′. The PCR reaction consisted of a hot-start incubation at 95°C for 2 min, followed by 95°C for 30 s, 56°C for 30 s,and 72°C for 30 s for 25 cycles. The PCR products were then analyzed by 2.0%

(w/v) agarose gel electrophoresis and detected under UV light by using ethidium bromide.

Analysis of *N*-linked glycosylation of ABCC11

The whole-cell lysates (150 μg of protein) were incubated with 190 U of either peptide *N*-glycosidase F (PNGase F; New England Biolabs, Ipswich, MA, USA) or endoglycosidase H (Endo H; New England Biolabs) at 37°C for 10 min before immunoblot analysis.

Immunoblotting detection of ABCC11 expressed in Flp-In-293 cells

The ABCC11 protein expressed in Flp-In-293 cells was detected by immunoblotting with the anti-ABCC11 polyclonal antibody. Briefly, samples were separated by SDS-PAGE (7.5% polyacrylamide slab gel) and transferred to Hy-bond ECL nitrocellulose membrane (GE Healthcare) by electroblotting at 15 V for 70 min. One hour after blocking in Tris-buffered saline containing 0.05% (v/v) Tween 20 (TTBS) and 5% (w/v) skim milk, immunoblotting was performed by using anti-human ABCC11 antibody (1:1000 dilution) as the first antibody and a goat anti-rabbit IgG-horseradish peroxidase (HRP)-conjugate (1:2000; Zymed, San Francisco, CA, USA) as the second antibody. HRP-dependent luminescence was developed by using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Boston, MA, USA) and detected with a Lumino Imaging Analyzer FAS-1000 (Toyobo, Osaka, Japan). To detect GAPDH, used as an internal loading control, immunoblot detection was carried out in the same manner as described above, except for the use of mouse monoclonal anti-GAPDH antibody (1:2000; American Research Products, Belmont, MA, USA) as the primary antibody and HRP-conjugated horse anti-mouse IgG (1:3000; Cell Signaling Technology, Inc., Beverly, MA, USA) as the second antibody.

Immunofluoresence microscopy

Flp-In-293 cells were replated onto 35-mm dishes at a concentration of 1.0×10^6 cells/dish. After 24 h, the cells were transfected with the ABCC11-pcDNA3.1/Hyg(−) vector by using Lipofectamine 2000 (Invitrogen), as described above. At 36 h after transfection, ABCC11-expressing Flp-In-293 cells were seeded onto collagen type I-coated coverslips and incubated under the above-mentioned culture conditions for a further 36 h. Cells were fixed with 4% paraformaldehyde in PBS at room temperature for 20 min. Thereafter, cell membranes were permeabilized by incubation with 0.02% Triton X-100 in PBS at room temperature for 5 min. To block the free aldehyde groups of formaldehyde, cells were treated with 10 mg/ml glycine in PBS at room temperature for 10 min, followed by a further incubation with 0.5% (w/v) bovine serum albumin (BSA) in PBS at room temperature for 1 h. To detect the ABCC11 protein, cells were treated with the anti-ABCC11 antibody (1:500 dilution) as the primary antibody and subsequently with the Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody (1:1000; Invitrogen). In the same preparations, nuclear DNA was stained with 4 μg/ml propidium iodide in PBS containing 0.5% (w/v) BSA. The immunofluorescence of Flp-In-293 cells was detected with a laser-scanning confocal fluorescence microscope (IX70/Fluoview; Olympus, Tokyo, Japan).

Detection of SNP 538G>A in ABCC11 gene by SmartAmp method

Templates used for the SmartAmp (K.K. DNAFORM, Yokohama, Japan) detection of the SNP 538G>A in the *ABCC11* gene were prepared from genomic DNA samples that were incubated at 98°C for 3 min. After chilling on ice, the sample preparation (20 ng of genomic DNA) was added directly into the reaction mixture (total volume of 25 μl) containing 2.0 μM folding primer (FP), 2.0 μM turn-back primer (TP), 1.0 μM boost primer (BP), 0.25 μM of each outer primer (OP1 and OP2), 20 μM competitive probe (CP), 1.4 mM dNTPs, 5% DMSO, 20 mM Tris-HCl (pH 8.0), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$, 0.1% (v/v) Tween®20, 1:100,000 SYBR® Green I (Takara Bio Inc., Shiga, Japan), and 0.24 U/μl *Aac* DNA polymerase (K.K. DNAFORM). SmartAmp reaction mixtures were incubated at 60°C for 60 min under an isothermal condition in a real-time PCR system (Mx3000P; Stratagene), where changes in the fluorescence intensity of SYBR Green I dye indicating DNA amplification were monitored during the reaction.

Statistical tests

Experimental data were analyzed by determining the statistical significance according to Student's *t* test.

RESULTS

Localization of ABCC11 in secretory cells of human ceruminous apocrine glands

Fig. 1*A* displays photomicrographs of wet- and dry-type ceruminous apocrine glands stained with hematoxylin-eosin. In this study, we obtained the samples from Japanese subjects carrying the heterozygous (538G/A) and SNP homozygous (538A/A) alleles. Wet-type ceruminous apocrine glands in the subjects carrying heterozygous (538G/A) alleles are well developed, and they exhibited large luminal cavities as compared with dry-type ceruminous apocrine glands (homozygous 538A/A). Such morphological differences were consistent with previously reported observations for wet and dry types of human ceruminous glands (23).

To elucidate the expression and cellular localization of the ABCC11 protein in the ceruminous apocrine gland, we developed an ABCC11-specific polyclonal antibody raised against the epitope encoding amino acid residues 746–804 in the ABCC11 protein. The specificity and immunological activity of this antibody were confirmed as described below. Immunofluorescence staining of human tissue specimens containing cerumen glands revealed the expression of ABCC11 in the luminal domain of the plasma membrane of secretory cells in both wet- and dry-type ceruminal glands (Fig. 1*B*). It is noteworthy, however, that ABCC11 was predominantly localized in intracellular granules and large vacuoles in the secretory cells of wet-type ceruminous glands (Fig. 1*B*). In contrast, such granular and vacuolar localization of ABCC11 was not detected in the dry-type ceruminous glands (Fig. 1*B*). It is likely that the nonsynonymous SNP 538G>A (Gly180Arg) greatly affects the cellular localization of the ABCC11 protein in secretory cells.

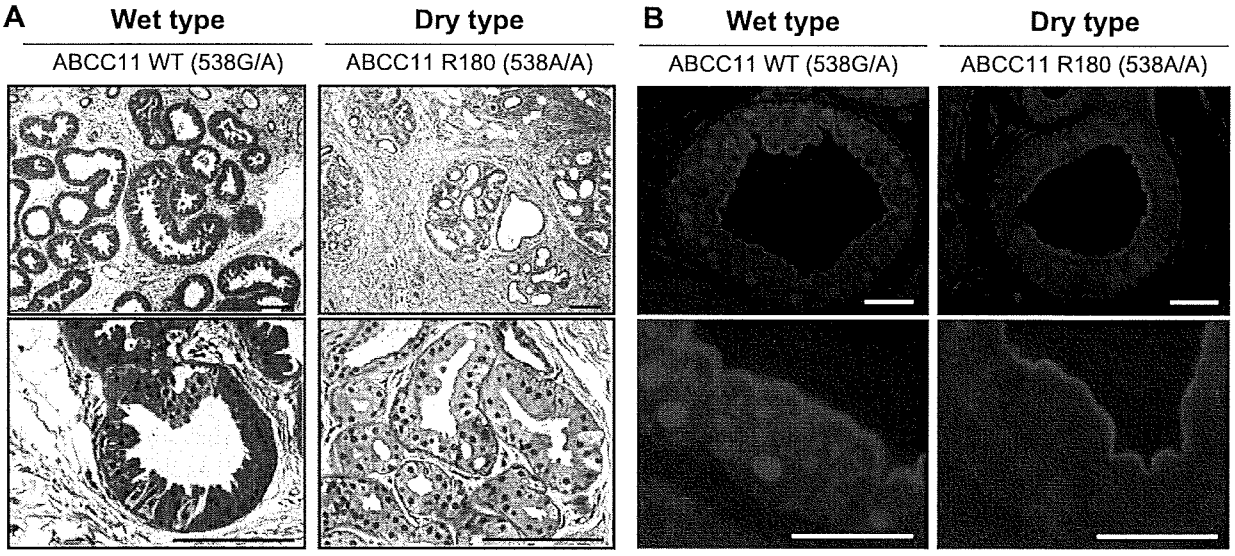


Figure 1. Microscopic pictures of wet- and dry-type ceruminous apocrine glands after hematoxylin-eosin staining and immunofluorescence staining of human ABCC11 protein. *A*) External auditory canal tissues were fixed with 4% paraformaldehyde in PBS (pH 7.4) overnight at 4°C, embedded in paraffin, and sectioned at 4 μm in thickness. Tissue sections were then stained with hematoxylin and eosin. *B*) Immunofluorescence staining of human ABCC11 protein in tissue specimens containing ceruminous apocrine glands, from human subjects carrying heterozygous (538G/A) and SNP homozygous (538A/A) alleles. ABCC11 protein expressed in secretory cells of wet- and dry-type ceruminous apocrine glands, 538G/A and 538A/A, was immunologically detected with ABCC11-specific polyclonal antibody and Alexa Fluor 488 (green). Cellular nuclei were stained with Hoechst 33342 (blue). Scale bars = 100 μm (*A*); 40 μm (*B*).

Validation of the ABCC11-specific antibody

Fig. 2 provides evidence that the polyclonal antibody is specific to human ABCC11. We expressed ABCC11 WT in Sf9 insect cells and Flp-In-293 human embryonic kidney cells by using pFastBac1 and pcDNA3.1/Hygro(−) vectors, respectively. Immunoblotting performed with the polyclonal antibody revealed one immunologically positive band (Fig. 2*C*, left column, arrowhead) in the ABCC11-expressing Sf9 cells. The molecular weight of the protein band was estimated to be ~150,000 (Fig. 2*C*). On the contrary, in the case of Flp-In-293 cells, two immunologically positive bands were observed at molecular weights of 180,000 and 150,000 (Fig. 2*C*, right column). After the sample was treated with PNGase F, the larger band (MW=180,000) disappeared, and one single im-

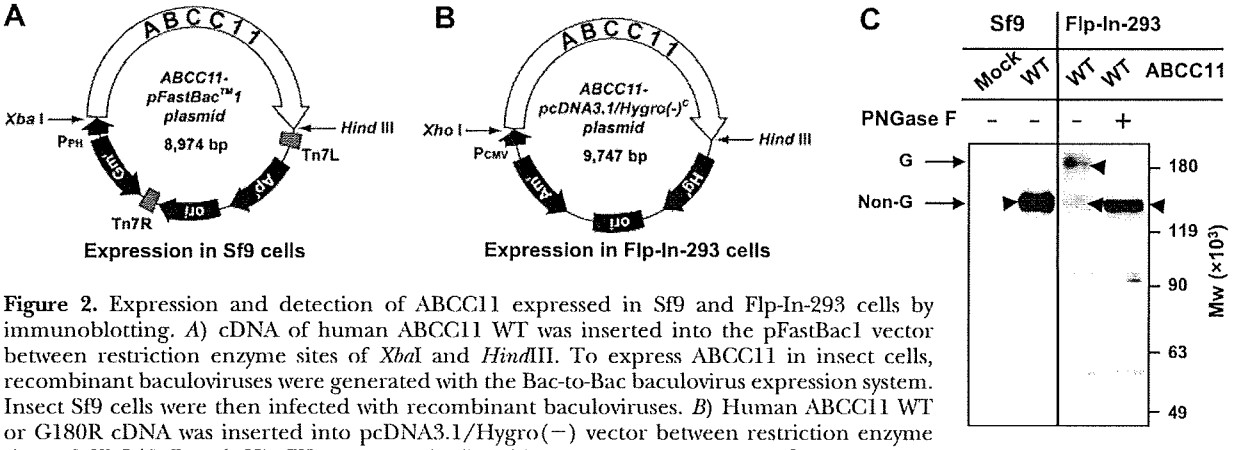


Figure 2. Expression and detection of ABCC11 expressed in Sf9 and Flp-In-293 cells by immunoblotting. *A*) cDNA of human ABCC11 WT was inserted into the pFastBac1 vector between restriction enzyme sites of *Xba*I and *Hind*III. To express ABCC11 in insect cells, recombinant baculoviruses were generated with the Bac-to-Bac baculovirus expression system. Insect Sf9 cells were then infected with recombinant baculoviruses. *B*) Human ABCC11 WT or G180R cDNA was inserted into pcDNA3.1/Hygro(−) vector between restriction enzyme sites of *Xho*I/*Sal*I and *Hind*III, respectively. Resulting expression construct [ABCC11 WT-pcDNA3.1/Hygro(−)] was used as template for site-directed mutagenesis with corresponding primers (Supplemental Tables 1 to 3). Human Flp-In-293 cells were transfected with expression vector by using Lipofectamine 2000 to express ABCC11 in cells. *C*) ABCC11 protein expressed in Sf9 and Flp-In-293 cells was detected by immunoblotting with anti-ABCC11 polyclonal antibody. ABCC11 protein expressed in Sf9 cells was not glycosylated, whereas it was *N*-link glycosylated in Flp-In-293 cells. The *N*-linked glycosylated form of ABCC11 (MW=180,000) was changed to the nonglycosylated form (MW=150,000) by PNGase F treatment.

munologically positive band remained to be detected at the molecular weight of 150,000 (Fig. 2*C*). This molecular weight is consistent with that estimated from the ABCC11 cDNA. Thus, these observations strongly suggest that the ABCC11 WT protein is *N*-glycosylated in mammalian cells.

Expression of ABCC11 WT and SNP variant in Flp-In-293 cells

To understand the molecular mechanism underlying the difference in cellular localization of the ABCC11 WT and the SNP variant (Fig. 1*B*), we expressed both types (WT and R180) in Flp-In-293 cells *in vitro*. **Figure 3** shows the differential interference and immunofluorescence images of Flp-In-293 cells expressing ABCC11 WT and R180 variant proteins, as well as Flp-In-293 cells transfected with a mock vector. These ABCC11 proteins were probed with the polyclonal antibody and then labeled with green fluorescence dye (Alexa Fluor 488), whereas DNA in the nuclei was stained with propydium iodide (red fluorescence). Strong green fluorescence was observed at the plasma membrane and within intracellular compartments in Flp-In-293 cells expressing ABCC11 WT. In the case of the SNP variant, however, expression of the ABCC11 R180 variant at the plasma membrane was negligibly low. In contrast, the variant protein was detected within intracellular compartments proximal to the cellular nuclei in Flp-In-293 cells.

Protein expression levels of ABCC11 WT and SNP variant and their *N*-linked glycosylation status

In accordance with the results shown in Fig. 3, immunoblotting experiments revealed a significant difference in the protein expression level between the WT (G180) and the SNP variant (R180) of ABCC11 (**Fig. 4*A***). Despite having similar mRNA levels, the expression level of the R180 variant protein was much lower than that of the WT (Fig. 4*A*). Interestingly, the R180 protein level was remarkably (14-fold) enhanced by treatment of the cells with the proteasome inhibitor MG132 (Fig. 4*B*). In contrast, the protein level of the WT was only moderately affected by MG132 treatment (Fig. 4*B*), suggesting that the R180 protein is more susceptible to proteasomal degradation than the WT. Furthermore, the R180 protein was not the *N*-linked glycosylated form, whereas the WT was expressed as both *N*-linked glycosylated and nonglycosylated forms (Fig. 4*C*). The *N*-linked glycan was digested by PNGase F, but not by Endo H (Fig. 4*D*).

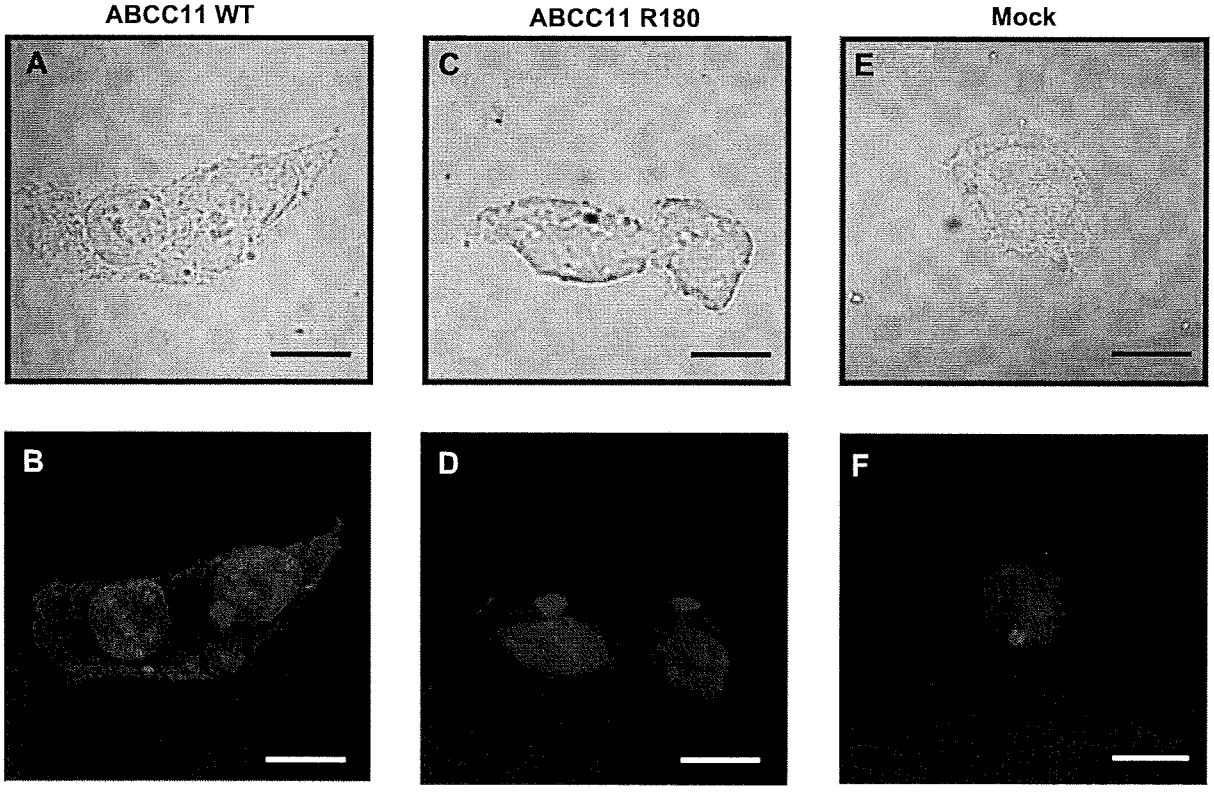


Figure 3. Immunocytochemical staining of Flp-In-293 cells expressing ABCC11 WT and R180 variant proteins. Differential interference (*A, C, E*) and immunofluorescence images (*B, D, F*). ABCC11 proteins were immunologically detected with ABCC11-specific polyclonal antibody and Alexa Fluor 488 (green). Cellular nuclei were stained by propydium iodide (red). Antibody interacts with epitope peptide residing in cytoplasmic domain (aa 746–804) between TM6 and TM7. Since Gly180 or Arg180 residue resides in TM1, this amino acid alteration (Gly180Arg) does not affect immunoreactivity. Scale bars = 10 μm.

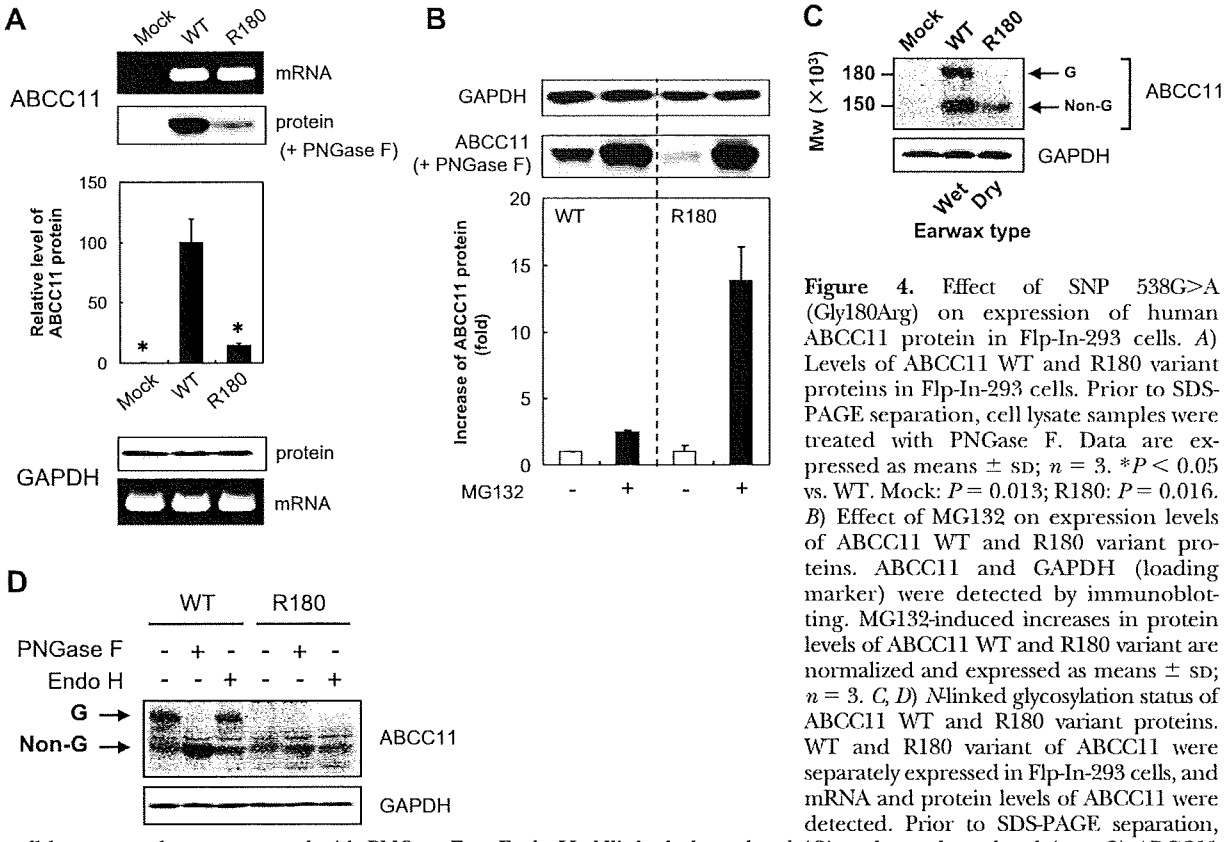


Figure 4. Effect of SNP 538G>A (Gly180Arg) on expression of human ABCC11 protein in Flp-In-293 cells. *A*) Levels of ABCC11 WT and R180 variant proteins in Flp-In-293 cells. Prior to SDS-PAGE separation, cell lysate samples were treated with PNGase F. Data are expressed as means ± SD; $n = 3$. $^*P < 0.05$ vs. WT. Mock: $P = 0.013$; R180: $P = 0.016$. *B*) Effect of MG132 on expression levels of ABCC11 WT and R180 variant proteins. ABCC11 and GAPDH (loading marker) were detected by immunoblotting. MG132-induced increases in protein levels of ABCC11 WT and R180 variant are normalized and expressed as means ± SD; $n = 3$. *C, D*) *N*-linked glycosylation status of ABCC11 WT and R180 variant proteins. WT and R180 variant of ABCC11 were separately expressed in Flp-In-293 cells, and mRNA and protein levels of ABCC11 were detected. Prior to SDS-PAGE separation, cell lysate samples were treated with PNGase F or Endo H. *N*-linked glycosylated (G) and nonglycosylated (non-G) ABCC11 proteins were detected by immunoblotting. GAPDH, a control for equal loading, was detected in a similar manner.

Identification of *N*-linked glycosylation sites in ABCC11 WT protein

Based on the amino acid sequence of ABCC11, a total of 8 potential *N*-linked glycosylation sites (Asn-*X*-Thr/Ser) was predicted (**Fig. 5*A***). The ABCC11 protein has 12 transmembrane helices, and its hydropathy profile indicates that only Asn838 and Asn844 reside in an extracellular loop between transmembrane helices TM7 and TM8 (Fig. 5*A*). Thus, we changed these Asn residues to Gln by site-directed mutagenesis and then expressed the protein in Flp-In-293 cells. Substitution of both Asn838 and Asn844 to Gln residues completely diminished *N*-linked glycosylation of the ABCC11 WT (Fig. 5*B*), demonstrating that these two Asn residues are *N*-linked glycosylation sites in the ABCC11 WT protein.

We hypothesized that amino acid substitution at 180 in the first transmembrane helix (TM1) might affect *N*-linked glycosylation of ABCC11. To examine our hypothesis, we substituted Gly180 to Arg, Lys, His, Asp, Glu, AL, and Pro in the ABCC11 WT protein. The substitution of Gly180 to a positively or negatively charged amino acid (*i.e.*, Arg, Lys, His, Asp, or Glu) diminished *N*-linked glycosylation of ABCC11, whereas substitution to a neutral amino acid (*i.e.*, AL or Pro) had no great effect (Fig. 5*C*). It is suggested that the electrostatic charge (either positive or negative) at aa 180 in the TM1 interferes with correct folding of the *de novo* synthesized ABCC11 protein in the ER.

Effect of nonsynonymous SNPs and a Δ27 mutation on the *N*-linked glycosylation of ABCC11

Furthermore, we created previously known nonsynonymous SNP variants and the Δ27 mutant of ABCC11 (**Fig. 6*A***) and expressed them in Flp-In-293 cells to examine their *N*-linked glycosylation status. The rare deletion mutation Δ27 results from the removal of a 9-aa stretch (Asp1313–Arg1321) from the C-terminal intracellular region of the ABCC11 protein (6). Both the rare mutation and G180R provide the dry type of earwax (6). Among the variants tested, only G180R and Δ27 diminished *N*-linked glycosylation of ABCC11 (Fig. 6*B*). Like the SNP variant R180 (Fig. 4*A, B*), the expression level of the Δ27 mutant protein was low (Fig. 6*C*), whereas it was significantly enhanced by MG132 treatments (Fig. 6*D*). Thus, lack of *N*-linked glycans of ABCC11 appears to be related with the dry type of human earwax.

Clinical genotyping of the SNP 538G>A (Gly180Arg) in the ABCC11 gene by the SmartAmp method

We tried to create a clinical method to genotype the SNP 538G>A in the heman *ABCC11* gene. For rapid genotyping, we have recently developed the SmartAmp method

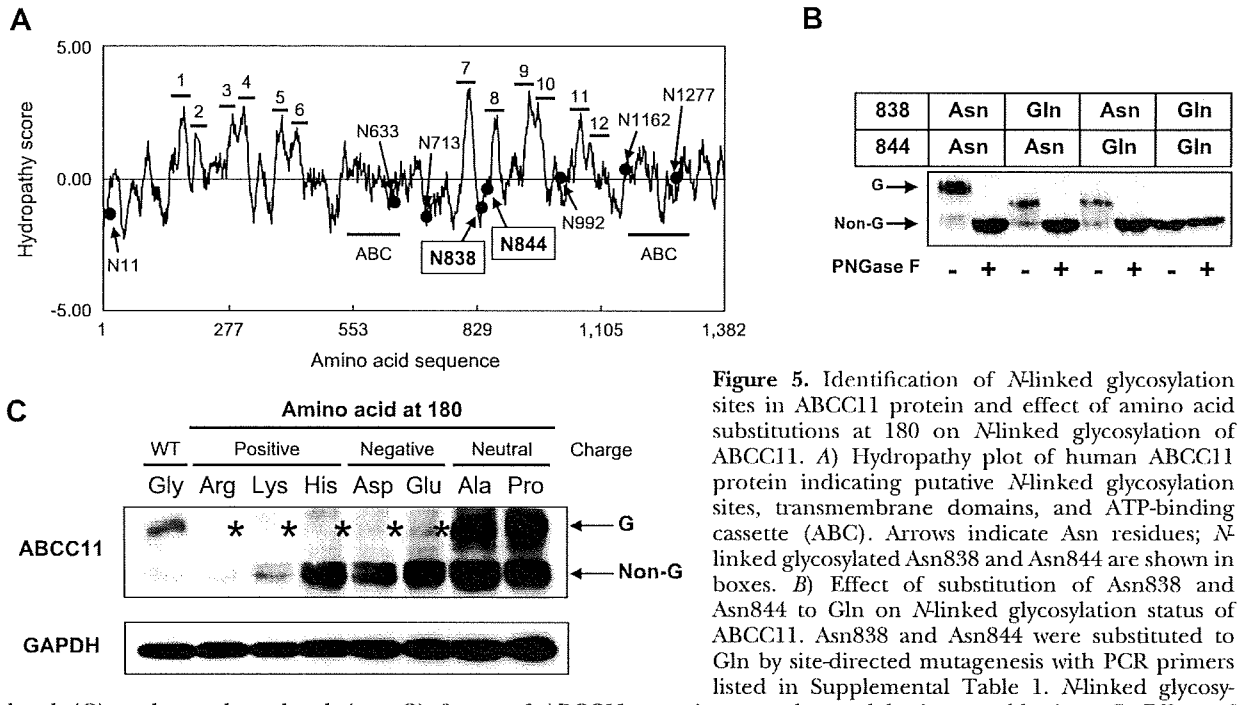


Figure 5. Identification of *N*-linked glycosylation sites in ABCC11 protein and effect of amino acid substitutions at 180 on *N*-linked glycosylation of ABCC11. *A*) Hydropathy plot of human ABCC11 protein indicating putative *N*-linked glycosylation sites, transmembrane domains, and ATP-binding cassette (ABC). Arrows indicate Asn residues; *N*-linked glycosylated Asn838 and Asn844 are shown in boxes. *B*) Effect of substitution of Asn838 and Asn844 to Gln on *N*-linked glycosylation status of ABCC11. Asn838 and Asn844 were substituted to Gln by site-directed mutagenesis with PCR primers listed in Supplemental Table 1. *N*-linked glycosylated (G) and nonglycosylated (non-G) forms of ABCC11 protein were detected by immunoblotting. *C*) Effect of electrostatic charges at amino acid residue 180 on *N*-linked glycosylation status of ABCC11. Gly180 residue in ABCC11 WT protein was substituted to Arg, Lys, His, Asp, Glu, AL, and Pro by site-directed mutagenesis with PCR primers listed in Supplemental Table 2. ABCC11 protein was detected by immunoblotting; glycosylation forms are indicated. Asterisk (*) indicates no *N*-linked glycosylation of ABCC11.

that enables us to detect genetic polymorphisms or mutations in ~30 min under isothermal conditions (24). **Figure 7*A*** schematically illustrates the strategy of SNP detection by the SmartAmp method. To determine the SNP 538G>A (Gly180Arg) in the *ABCC11* gene, we prepared one set of primers designated TP, FP, BP, OP, and CP (Fig. 7*B*). The TPs discriminate the polymorphism 538G or 538A in the *ABCC11* gene, and the CPs

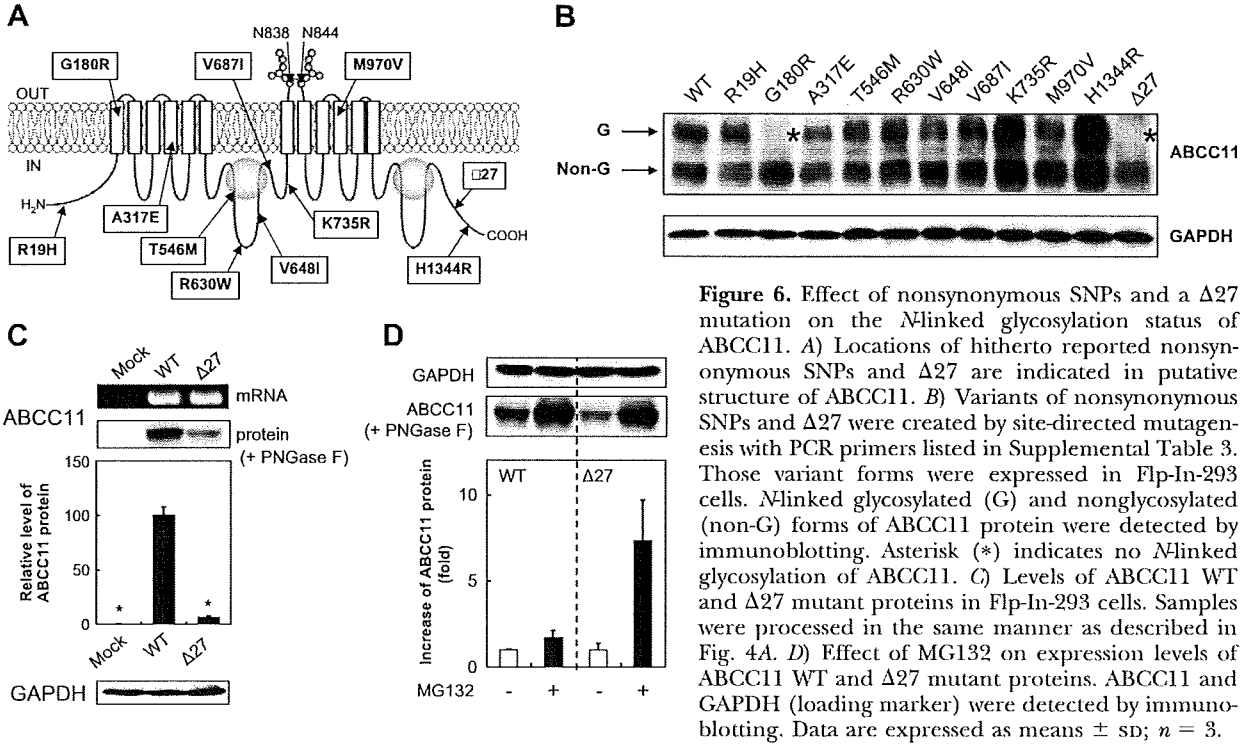


Figure 6. Effect of nonsynonymous SNPs and a Δ27 mutation on the *N*-linked glycosylation status of ABCC11. *A*) Locations of hitherto reported nonsynonymous SNPs and Δ27 are indicated in putative structure of ABCC11. *B*) Variants of nonsynonymous SNPs and Δ27 were created by site-directed mutagenesis with PCR primers listed in Supplemental Table 3. Those variant forms were expressed in Flp-In-293 cells. *N*-linked glycosylated (G) and nonglycosylated (non-G) forms of ABCC11 protein were detected by immunoblotting. Asterisk (*) indicates no *N*-linked glycosylation of ABCC11. *C*) Levels of ABCC11 WT and Δ27 mutant proteins in Flp-In-293 cells. Samples were processed in the same manner as described in Fig. 4*A*. *D*) Effect of MG132 on expression levels of ABCC11 WT and Δ27 mutant proteins. ABCC11 and GAPDH (loading marker) were detected by immunoblotting. Data are expressed as means ± SD; $n = 3$.

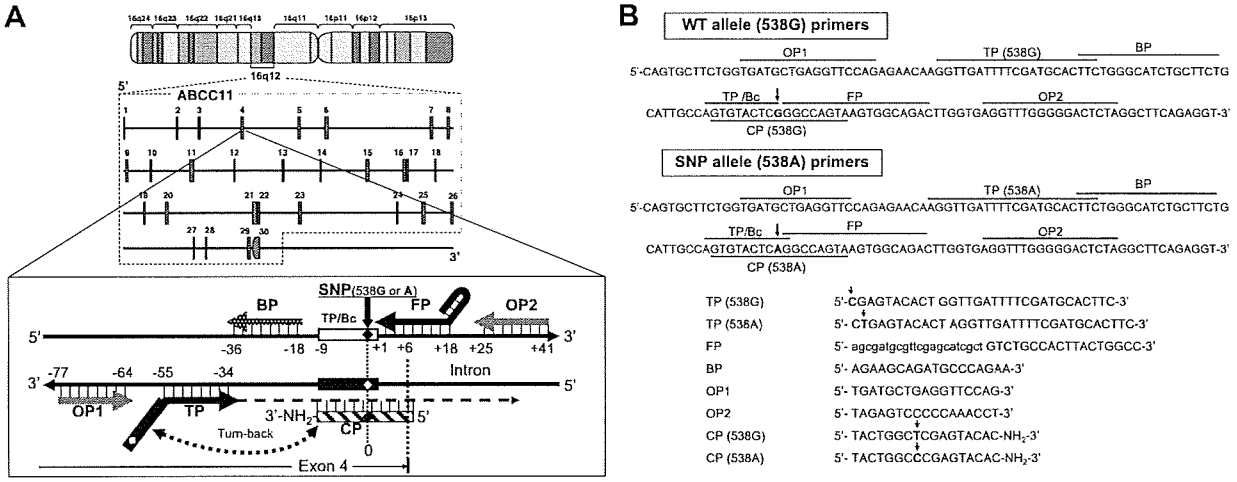


Figure 7. Strategy for SmartAmp-based detection of SNP 538G>A in *ABCC11* gene. *A*) Schematic illustration of SmartAmp-based SNP detection. SNP 538G>A resides in exon 4 of ABCC11 gene on chromosome 16q12. *B*) Partial genomic DNA sequences of *ABCC11* gene carrying WT (538G) and SNP (538A) alleles and sequences of primers used for SmartAmp assay.

inhibit the background amplification from mismatch sequence pairs (25, 26) (Fig. 7*A*). These primers selectively recognized the SNP 538G>A of the *ABCC11* gene to discriminate homozygous 538G/G (wet type), heterozygous 538G/A (wet type), and homozygous 538A/A (dry type) in genomic DNA (**Fig. 8*A***). By this new method, we typed the SNP in genomic DNA samples from a total of 124 healthy volunteers and compared the results obtained by DNA sequencing. Neither false positives nor false negatives were observed (Fig. 8*B*). Thus, the perfect matching validated the SmartAmp-based SNP typing method for use in clinical practice.

Since previous phenotype-based studies have suggested a positive association between the wet-type earwax and axillary osmidrosis (1), we analyzed the SNP in the *ABCC11* gene of Japanese axillary osmidrosis patients. In this clinical study, axillary osmidrosis was diagnosed basically from both the self-declaration of patients and the odor-smelling test carried out by authorized medical doctors. The earwax type was determined from wax samples obtained by stirring a cotton swab in the external auditory canal of the patient. **Table 1** summarizes the clinical results from this study with respect to the *ABCC11* genotype as well as axillary osmidrosis and earwax types. It is notable that all axillary osmidrosis patients carrying the 538 alleles as either WT homozygous (538G/G) or heterozygous (538G/A) have the wet type of earwax, without exception. Although the population size was rather small in this study, the *ABCC11* WT allele is suggested to be a genetic biomarker for clinical diagnosis of axillary osmidrosis.

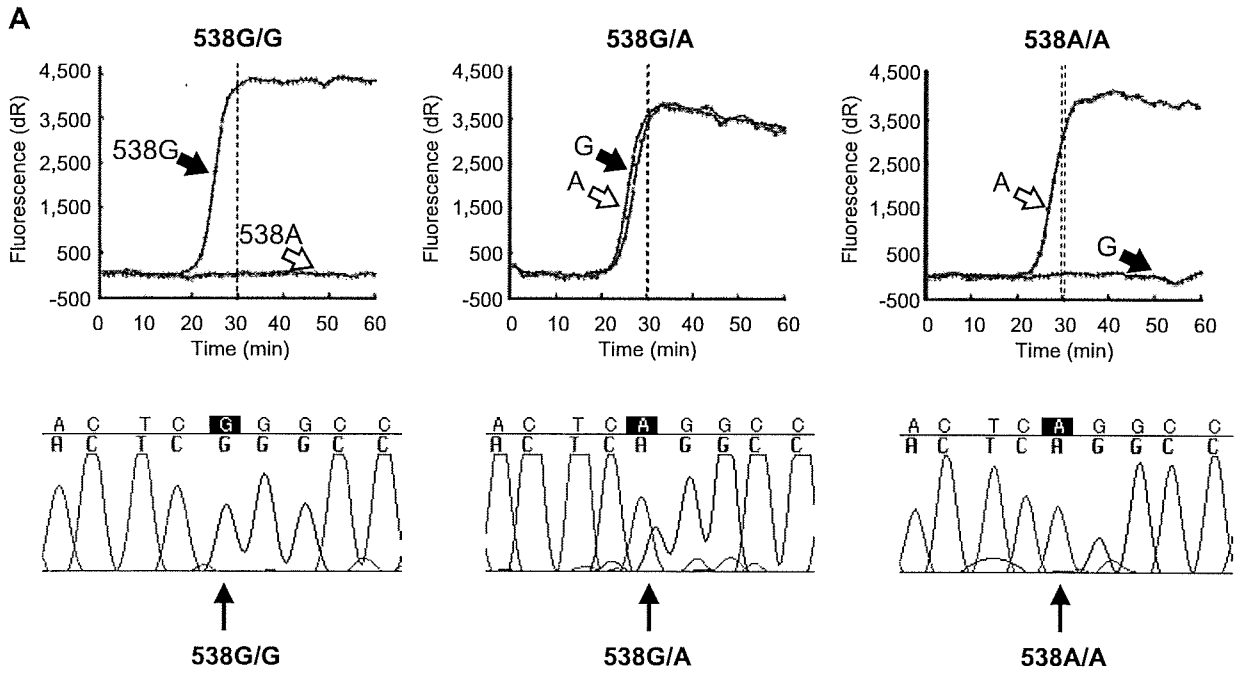
DISCUSSION

Proteasomal degradation of ABCC11 R180 is the molecular mechanism determining the dry-type earwax

The present study provides direct evidence that human ABCC11 WT is an *N*-linked glycosylated protein, which is localized in intracellular granules and large vacuoles as well as at the luminal membrane of secretory cells in the cerumen apocrine gland. *N*-linked glycosylation occurs at both Asn838 and Asn844 in the extracellular loop between TM7 and 8 TM8 of the ABCC11 WT protein. In contrast, the SNP variant R180 lacks *N*-linked glycosylation and readily undergoes proteasomal degradation, most probably *via* ubiquitination. As a consequence, granular or vacuolar localization was not detected in the cerumen apocrine gland for the SNP variant.

Morphological differences were previously reported between the secretory cells of wet and dry types of human ceruminous glands (23). In the wet-type glands, the Golgi apparatus was reportedly well developed, whereas it was generally small in the corresponding cells of the dry type. Furthermore, intracellular granules were abundantly observed in the wet-type gland in close relationship to their well-developed Golgi apparatus, whereas intracellular granules were rare in the dry-type gland.

The ER and Golgi apparatus are the sites of synthesis and maturation of proteins destined for the plasma membrane, for the secretory and endocytic organelles, and for secretion (27, 28). Efficient quality-control systems have evolved to prevent incompletely folded proteins from moving along the secretory pathway. Accumulation of misfolded proteins in the ER would detrimentally affect cellular functions. Therefore, misfolded proteins may be removed from the ER by retrotranslocation to the cytosol compartment, where they are degraded by the ubiquitin-proteasome system. This process is known as endoplasmic reticulum-associated degradation (ERAD) (29-32). It is likely that both the SNP variant R180 and the Δ27 mutant are recognized as misfolded proteins in the ER and readily undergo the proteasomal degradation. Indeed, the protein levels of both R180 (Fig. 4*B*) and Δ27 (Fig. 6*D*) were greatly enhanced by treatment of cells with the



B

	SMAP			
Sequence	Geno - type	G/G	G/A	A/A
	G/G	4	0	0
	G/A	0	33	0
	A/A	0	0	87

N =124

Wet type Dry type

Figure 8. Diagnosis of SNP 538G>A by SmartAmp assay and DNA sequencing. *A*) Time course of SmartAmp assay reaction with *ABCC11* allele-specific primers. Results of SmartAmp assay (top panels) and DNA sequence analysis (bottom panels) are shown for 3 diploid genotypes of ABCC11. *B*) Comparison of SNP analysis data obtained by SmartAmp method and DNA sequencing.

proteasome inhibitor MG132. This protein processing may influence the activity of ceruminous apocrine glands and determine the type of human earwax. In **Fig. 9**, we schematically illustrate the effects of the SNP on the cellular localization and function of ABCC11 in secretory cells of the apocrine gland.

TABLE 1. *Genotyping of the ABCC11 gene in axillary osmidrosis patients and comparison with earwax type*

	ABCC11 genotype (538G>A)		
Diagnosis	G/G	G/A	A/A
Axillary osmidosis			
Positive	1	11	0
Negative	0	0	2
Earwax type			
Wet	1	11	0
Dry	0	0	2

Diagnosis of axillary osmidrosis and earwax type as well as genotyping of *ABCC11* were performed at Fujita Health University School of Medicine according to the protocol approved by the Ethical Review Board. The SNP 538G>A in the *ABCC11* gene was analyzed by both DNA sequencing and the SmartAmp method.

We have recently demonstrated that the intramolecular disulfide bond formation and *N*-linked glycosylation in the extracellular loop are important for stability of human ABC transporter ABCG2 in the ER (33-36). During *de novo* synthesis in the ER, cysteine disulfide bonds are formed, and oligosaccharides are added to asparagine (*N*-linked glycosylation) or serine residues (*O*-glycosylation) of glycoproteins. In general, *N*-linked glycans are added *en block* to proteins as "core oligosaccharides" ($Glc_3Man_9GlcNAc_2$) (27, 29). As described above, Asn838 and Asn844 are glycosylation target sites in human ABCC11. The *N*-linked glycans are thought to be subjected to extensive modification as glycoproteins mature and move through the ER *via* the Golgi apparatus to their final destination, for example, intracellular granules and large vacuoles of secretory cells in the apocrine gland. It is puzzling, however, how amino acid alteration at 180 in TM1 affected the *N*-linked glycosylation occurring in the extracellular loop between TM7 and TM8. To answer this question, we assume that the electrostatic charge at aa 180 in the TM1 could interfere with correct folding of the *de novo* synthesized ABCC11 protein in the ER, as shown in Fig.

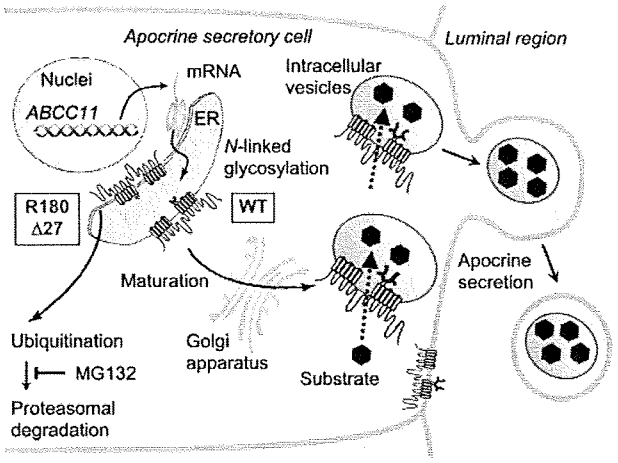


Figure 9. Schematic illustration of intracellular sorting of ABCC11 WT and proteasomal degradation of R180 and Δ27 variants in secretory cells in ceruminous apocrine gland. *De novo* synthesized ABCC11 WT is *N*-link glycosylated at Asn838 and Asn844 in the ER, further processed in the Golgi apparatus, and destined for membrane of intracellular granules and vacuoles. Ceruminous components are thought to be transported by ABCC11 WT and sequestrated in intracellular granules and vacuoles. SNP variant R180 and Δ27 mutant lacking *N*-linked glycosylation are recognized as misfolded proteins in the ER and readily undergo ubiquitination and proteasomal degradation.

5C. It is important to note, however, that *N*-linked glycosylation may affect in part the protein stability, but it is not the principal factor for correctly folding and/or enhancing the stability of the ABCC11 protein in the ER. As exemplified by our recent study, substitutions of one single amino acid due to nonsynonymous polymorphisms greatly affected the protein stability of SNP variants of human ABCG2 (33), whereas deletion of *N*-linked glycosylation at Asn596 reduced the protein expression level of ABCG2 WT only by half, without any change in its plasma membrane localization (unpublished results). Thus, molecular mechanisms underlying the misfolding and degradation of membrane proteins appear to be rather complex, as they involve interactions with different chaperone proteins.

At present, it remains unclear how misfolded membrane proteins are selected and destroyed during ERAD. Chaperones are considered to solubilize aggregation-prone motifs. In the case of the yeast ABC transporter Ste6p, a 12-transmembrane protein, it has recently been shown that Hsp70/40s act before ubiquitination and facilitate Ste6p association with an E3 ubiquitin ligase (37). Furthermore, polyubiquitination was a prerequisite for retrotranslocation, which required the Cdc48 complex and ATP (37). In this regard, Wang *et al.* (38) and Younger *et al.* (39) recently provided new insights into the complexity of protein networks that govern the fate of the cystic fibrosis transmembrane conductance regulator (CFTR), an apical membrane ABC transporter. The loss of phenylalanine residue at position 508 in the first nucleotide-binding domain disrupts the folding pathway of CFTR protein in the ER, and subsequently misfolded CFTR is targeted for ERAD (38-42). In the case of human bile salt export pump (BSEP/ABCB11), at least two *N*-linked glycans are reportedly important for protein stability, intracellular trafficking, and function in the apical membrane (43). In this context, it is increasingly important to identify and to characterize multiple chaperone proteins that control the folding and degradation of ABC transporter proteins, including ABCC11.

Clinical relevance of genetic polymorphisms of human ABCC11

Genetic polymorphisms of human ABC transporter genes are reportedly related to the risk of disease and patient response to medication (44, 45). Phenotype-based studies have suggested positive associations among the wet-type earwax, axillary osmidrosis (1), colostrum secretion from the mammary gland (2), and the potential susceptibility of breast cancer (4). Our genotyping study has revealed that the *ABCC11* WT allele is intimately associated with axillary osmidrosis (**Table 1**). Since the frequency of the *ABCC11* WT allele is low in Japan, axillary osmidrosis is recognized as a disease that is covered by the national health insurance system. Axillary osmidrosis is often perceived, especially by young women, as a distressing and troublesome problem.

Sweat produced by axillary glands is odorless. Secretions from the apocrine glands, however, can be converted to odoriferous compounds by bacteria (*Corynebacteria*), which results in the formation of the unique "human axillary odor" (46). In axillary osmidrosis patients (G/G homozygote or G/A heterozygote), significantly numerous and larger-sized axillary apocrine glands were observed as compared with the subjects carrying the A/A homozygote. Our results (**Table 1**) suggest that the 538G allele of the *ABCC11* gene is associated with axillary osmidrosis and that ABCC11 WT is responsible for the secretion of preodoriferous compounds from the axillary apocrine gland.

In primates, the axillary odors may play a role in olfactory communication, although no documented behavioral or endocrine changes by volatiles produced in the axillae have been reported to occur in humans. Previous studies have shown that androgen steroids were present in the axillary area. Androsterone sulfate (AS) and DHEAS were detected in the extract of axillary hairs, in addition to high levels of cholesterol (47). It was also demonstrated, following injection of radioactive pregnenolone or progesterone, that steroid secretion was concentrated in the axillary area (48). The axillary sweat collected in these studies from the skin surface, however, represents a mixture of materials from apocrine, eccrine, and sebaceous glands, in addition to desquamating epidermal cells. In this respect, Lebows *et al.* (49) conclusively demonstrated that at least two androgen steroids, AS and DHEAS, in addi-

tion to cholesterol, did exist in pure apocrine secretions. It has more recently been reported that DHAES is transported by ABCC11 WT (13-15). Further studies are needed to define more precisely the substrates for ABCC11 in apocrine glands.

CONCLUSIONS

Based on the previous phenotype analysis, mortality and frequency rates for breast cancer were reported to be associated with the frequency of the allele for wet-type earwax (4), although the association with breast cancer was controversial (50). Our preliminary genotyping study, however, revealed that the allele of 538G (wild type) in the *ABCC11* gene is associated with risk of breast cancer among a total of 416 Japanese premenopausal women as compared with the 538A (SNP type) allele. The odds ratio was found to be 1.86 (unpublished results). The SNP typing method presented in this study would provide a practical tool to examine a latent genetic link between wet-type earwax and the potential risk of breast cancer. FJ

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A strong association of axillary osmidrosis with the wet earwax type determined by genotyping of the *ABCC11* gene

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Abstract

Background: Two types of cerumen occur in humans: the wet type with brownish, sticky earwax, and the dry type with a lack of or reduced ceruminous secretion. The wet type is common in populations of European and African origin, while the dry type is frequently seen in Eastern Asian populations. An association between axillary odor and the wet-type earwax was first identified approximately 70 years ago. The data were based on a phenotypical analysis of the two phenotypes among the Japanese by a researcher or by self-declaration of the subjects examined, and were not obtained using definite diagnostic methods. Recently, we identified a single-nucleotide polymorphism (SNP; rs17822931) of the *ABCC11* gene as the determinant of the earwax types. In the present study, to determine whether the SNP can serve as a diagnostic marker for axillary osmidrosis (AO), we examined genotypes at rs17822931 in 79 Japanese AO individuals. AO was defined here as a clinical condition of individuals with a deep anxiety regarding axillary odor and had undergone the removal of bilateral axillary apocrine glands.

Results: A comparison of the frequencies of genotypes at rs17822931 in the 79 AO individuals and in 161 Japanese from the general population showed that AO was strongly associated with the wet earwax genotype. A total of 78 (98.7%) of 79 AO patients had either the GG or GA genotype, while these genotypes were observed in 35.4% (57/161) of the subjects from the general population ($p < 1.1 \times 10^{-24}$, by Fisher's exact test).

Conclusion: The strong association between the wet-earwax associated *ABCC11*-genotypes (GG and GA) and AO identified in this study indicates that the genotypes are good markers for the diagnosis of AO. In addition, these results suggest that having the allele G is a prerequisite for the axillary odor expression. In other words, the ABCC11 protein may play a role in the excretory function of the axillary apocrine gland. Together, these results suggest that when an AO individual visiting a hospital is diagnosed with dry-type earwax by *ABCC11*-genotyping, surgical removal of their axillary glands may not be indicated.

Background

Apocrine and/or eccrine glands in the human body cause odor, especially from the axillary and pubic apocrine glands. As in other mammals, the odor may have a pheromone-like effect on the opposite sex. Although the odor does not affect health, axillary osmidrosis (AO) is a condition in which an individual feels uncomfortable with their axillary odor, regardless of its strength, and may visit a hospital. Surgery to remove the axillary gland may be performed on demand. AO is likely an oligogenic trait with rs17822931 accounting for most of the phenotypic variation and other unidentified functional variants accounting for the remainder. However, no definite diagnostic criteria or objective measuring methods have been developed to characterize the odor, and whether an individual suffers from AO depends mainly on their assessment and/or on examiner's judgment. Human body odor may result from the breakdown of precursors into a pungent odorant by skin bacteria [1], but it is unclear if AO is this type of odor.

The consistency of human earwax is a dimorphic genetic trait and two distinct types are known: the wet type characterized as sticky, brownish earwax, and the dry type characterized as scurf or scales of the external ear canal. The wet type is completely dominant to the dry type, and is very common in populations of European and African origin (~95% and ~100%, respectively) [2-7]. In contrast, the dry type is frequently seen in Eastern Asian populations, with the prevalence of the wet earwax being ~15% in Japan, ~5% in Korea and ~10% among the Han Chinese [2,7]. We have recently identified an earwax determining SNP, c.538G>A (rs17822931), in the *ABCC11* gene [6], and confirmed the gene as an earwax-type determinant. We calculated the allele frequencies in various ethnic populations [6], which can now be retrieved from existing databases. From the HapMap data, the G-allele (defining the wet type) frequency is estimated to be 1.000 in the Yoruba population (Africa), 0.875 in CEPH families (Europe), and 0.111 in Tokyo habitants (Japan), and the frequencies estimated from ALFRED (the allele frequency database) show overall accordance with those from the HapMap data.

A relationship between axillary odor and the wet-type earwax was first noticed among the Japanese population concurrent with the first discovery of the earwax type as a Mendelian trait. Japanese clinicians assert an association between axillary odor and earwax type; however, since no definite diagnostic criteria or measuring methods were available for the two traits, the data is based on observations of the two respective traits.

Here we report the result of a genotyping study examining rs17822931 of the *ABCC11* gene in Japanese individuals with AO, and discuss the ABCC11 genotype as a diagnostic tool for AO.

Results

We analyzed a total of 79 AO individuals from either Nagasaki or Okinawa prefectures. Of the 79 AO patients, 5 were GG homozygotes, 73 were GA heterozygotes, and 1 was an AA homozygote. Therefore, 98.7% (78/79) of the AO individuals had the GG or GA genotype (Table 1). In contrast, the GG and GA genotypes were observed in 35.4% (57/161) of the overall population in the prefec-

Table 1: Association of AO with the wet earwax type

Subject studied	Genotype at the rs17822931 locus (earwax phenotype)					
	GG	GA	(wet type)	AA	(dry type)	total
Individuals with AO (Kyushu)	5	73	(78)[a]	1	(1)[a]	79
General habitants in Kyushu	6	51	(57)[a]	104	(104)[a]	161
total			(135)[a]		(105)[a]	240
Individuals with AO (Okinawa)	3	34	(37)[b]	1	(1)[b]	38
General habitants in Okinawa	2	15	(17)[b]	17	(17)[b]	34
total			(54)[b]		(18)[b]	72
Individuals with AO (Nagasai)	2	39	(41)[c]	0	(0)[c]	41
General habitants in Nagasaki	4	36	(40)[c]	87	(87)[c]	127
total			(81)[c]		(87)[c]	168

[a]$p < 1.1 \times 10^{-24}$, [b]$p < 3.0 \times 10^{-6}$, [c]$p < 8.4 \times 10^{-17}$: all comparisons were performed under a dominant model. All statistical analyses were done by Fisher's exact test. Hardy-Weinberg equilibrium was supported under the observed allele frequency in control samples by Exact Hardy-Weinberg test (p-value > 0.1) [18]. These comparisons were performed using plink software [19]http://pngu.mgh.harvard.edu/purcell/plink/.

tures. In Nagasaki, GG, GA and AA genotypes are observed in 2, 39 and none of the 41 AO individuals, and in 4, 36 and 87 of the general population samples, respectively. Likewise in Okinawa prefecture, GG, GA and AA genotypes comprised 3, 34, and 1 of the AO patients, and 2, 15, and 17 of the general Okinawans population sample. Fisher's exact test showed a strong association between the wet type genotype and AO ($p < 8.4 \times 10^{-17}$ for the Nagasaki habitants, and $p < 3.0 \times 10^{-6}$ for the Okinawans) (Table 1). Although G allele frequency is considerably higher among Okinawans than habitants in other Kyushu areas [6], no significant difference was detected between general Nagasaki and Okinawa populations ($p > 0.06$). This may be due to the relatively small number of samples from Okinawa in this study. Fisher's exact test of the combined data showed a strong association of the wet type genotype and AO ($p < 1.1 \times 10^{-24}$).

Discussion

We have shown that AO in the Japanese population is strongly associated with the wet earwax genotypes, with the results supporting the 70-year-old data of a strong, positive association between the two traits. If all AO in the Japanese is a Mendelian trait and is primarily determined by allele G at the c.538G/A polymorphic site in the *ABCC11* gene, an all-or-none result would have been expected. In other words, under this condition, AA homozygotes should not have been included in the AO group, and all individuals with GG or GA genotype should have AO. However, a single individual with the AA genotype was present in our series of AO samples, and, thus, not all the samples showed deterministic association (Table 1). Since no objective way to quantify or qualify axillary odor is available and the diagnosis of any given AO individual is made on the basis of their history and complaints, we focused in this study only on AO individuals who visited plastic surgery clinics and did not assess the odor quantity of GG and GA individuals in the general population. Some individuals without AO may exist, and their axillary odor may be controlled by other genes and/or factors that modify the *ABCC11* function. Primary (cause unknown and possibly genetic) and secondary (multiple causes including anxiety, menopause, hyperthyroidism, stroke, drugs, amongst other causes) hyperhidrosis may be an explicable factor for AO in individuals with the AA genotype. It is plausible that the *ABCC11* gene primarily determines the quality of AO, while modifiers play a role in its quantity, such as pre-determination regarding the number of the apocrine glands in the axilla.

The *ABCC11* gene, which encodes MRP8, is expressed in various types of tissues [8,9] and is a member of the ATP-binding cassette transporter gene family [10]. Most ABC transporter proteins are localized to the plasma membrane and are ATP-dependent transporters of a broad range of compounds [11], such as cyclic nucleotides, lipophilic anions (glutathione-conjugated LTC4), sulfated steroids (DHEAS and $E_1$3S), glucuronides ($E_2$17βG), bile constituents (glycocholate and taurocholate), and monoglutamates (methotrexate) [12]. MRP8 is localized to the apical membrane of MDCK cells when expressed artificially [13]. Since most MRP proteins transport substrates from the inside to the outside of the cell, certain compound(s) that may cause axillary odor are secreted through MRP8 in the axillary apocrine gland. The axillary gland of individuals with the wet earwax type may secrete the materials more highly than that of the dry type individuals, as seen in a previous in vitro experiment [6].

The nature of axillary odor and whether the axillary odorants come directly from secreted materials of the axillary gland are unknown. Some carboxylic acids were reported to be possible components of such odorants. Zeng et al. [14] demonstrated that (*E*)-3-methylhex-2-enoic acid (3M2H) is a key odorant component, and its hydrated analogue (*RS*)-3-hydroxy-3-methylhexanonoic acid (HMHA) was the most abundant pungent odorant in the axilla [15]. Sweat itself does not smell, but skin bacteria (*Corynebacteria*) transform non-odoriferous precursors in sweat into a pungent odorant [1]. In addition, a specific Zn-dependent *N*-acyl-glutamine aminoacylase (N-AGA) in the bacteria has been reported to catalyze a reaction that produces 3M2H and HMHA from *N*-acyl-glutamine conjugates secreted into sweat in the axilla [15]. Natsch et al. [16] claimed that since there are other odoriferous materials, the proportion of these components causes odor variance among individuals. However, since axillary odor can be detected immediately after sweating (especially a large amount of rapid nervous sweating), there is insufficient time for bacterial growth. In addition, since the odor does not completely disappear by washing with water, but disappears with the use of soap, axillary odorants may contain certain lipophilic components, as does earwax. Therefore, it remains unclear whether these precursors in sweat are substrates of MRP8 and their secretion might be reduced or lacking in individuals with dry type earwax.

Recently, we examined the biochemical characteristics of the G allele (wild type allele) and A allele (mutant allele) [17]. Our results showed that the wild type ABCC11 protein is glycosylated and localized to the ceruminous gland membrane, but mutant ABCC11 from the A allele is not glycosylated and is degraded rapidly by the proteasome system. Degradation by the proteasome is not a complete process and some of the protein is localized on the cell surface membrane; therefore, mutant ABCC11 may retain some excretion function [17]. In this context, body odorant derived from ABCC11 function may represent a quantitative trait that depends on the protein levels on the cell

surface. Previously, we reported on the excretion properties of wild type and mutant ABCC11 using cGMP as a substrate. Since cGMP is not an authentic substrate for odor, further work is needed to identify the odorant in AO or from the ABCC11 substrate not only for biochemical characterization, but also as an objective tool to measure the axillary odor

Conclusion

In the present study, we demonstrated a strong association between the wet earwax genotype and AO. Our results suggest that genotyping at the rs17822931 locus may be a useful tool for supplementing the diagnosis of patients that present at clinic with OA. A result of this study suggests that the presence of allele G at the rs17822931 locus is a prerequisite for AO. Since almost all of the patients complaining of AO in this study did not have the AA genotype (78/79), we suggest that further study may prove rs17822931 to provide useful additional information in diagnosing AO patients. Two key issues remain to be addressed. The first is that, although the estimated sensitivity of the genotype diagnostic test from this study is high (approximately 99%) for patients who present with AO, the specificity of the test in this context is low, with 35% of controls also carrying the G risk allele. Second, it is vital that further research identify more objective clinical definitions of AO, since the sensitivity and specificity data presented in this study are conditional upon the subjective diagnosis of AO.

Methods

Subjects studied

The examinees included 79 Japanese individuals with AO, who were examined at plastic surgery clinics. Of the 79 AO samples, 41 were from Nagasaki prefecture and the remaining 38 were from Okinawa prefecture. Both prefectures are located in the Kyushu area, the most western district of Japan. Wet earwax frequency is different among prefectures of Japan [2], so we divided the samples into two groups based on the prefectures. One hundred twenty seven samples in Nagasaki and 34 samples in Okinawa were used as general population controls for chi-square test. These samples were previously collected for calculating the allele frequency of rs17822931 in ABCC11. All of the samples from AO cases and controls were collected with written informed consent, and protocols for the present study were approved by the Committee for the Ethical Issues on Human Genome and Gene Analysis at Nagasaki University.

In this study, AO individuals were defined as those who were anxious about axillary odor and had received a surgical operation in the clinics to remove their axillary apocrine glands. In general, some Japanese are very sensitive and nervous of body odor and often visit the clinics, probably because the majority of the population have faint odor. From this background, plastic surgeons are familiar with AO and the collection of AO patients is easy. However, no objective diagnostic methods are available for axillary odor. Therefore, diagnosis of AO was made through self-declaration by the individual and through the clinician's judgment at interview prior to the operation. Earwax type was not considered for the AO diagnosis. Although AO due to primary and secondary hyperhidrosis was excluded as much as possible, individuals with such conditions may have been included in our samples. If we collected samples from individuals with faint axillary odor (or without odor), well-trained plastic surgeons who collected the "AO" patient judged the axillary odor. In this situation, our association could be defined as a double blind study, but it is difficult to smell the axilla in the general population. Therefore, we focused our interest on the measurement the sensitivity of the earwax genotype to judge AO. When objective diagnostic methods are available for axillary odor, a complete double blind study will be feasible.

Genotyping at the SNP site (rs17822931) and association study

Genomic DNA was extracted from all examinees, and was subjected to PCR-based genotyping at the SNP site, rs17822931 (c.538G>A), in the *ABCC11* gene. For the TaqMan genotyping, VIC-labeled TaqMan MGB wet-probe (5'-CAGTGTACTCGGGCCAG-3') and FAM-labeled TaqMan MGB dry-probe (5'-CAGTGTACTCAGGCCAG-3') were used as hydrolyzing probes, while EW-ampF (5'-CTTCTGGGCATCTGCTTCTG-3') and EW-ampR (5'-CAAACCTCACCAAGTCTGCCA-3') were used as amplification primers. Reactions were carried out using TaqMan Universal PCR Master Mix (AppliedBiosystems). Homozygotes for allele A were categorized to have the dry earwax type and others the wet type [6]. The number of AO individuals within each respective genotype was statistically compared with that in the general population by Fisher's exact test, and Hardy-Weinberg equilibrium was tested under the observed allele frequency [18,19].

Authors' contributions

MN collected samples from the Nagasaki area and extracted DNA. NM extracted DNA from tissue samples of AO individuals and performed genotyping. AH participated in the design of the study, collected samples from the Okinawa area and analyzed the clinical data of AO patients. KY performed statistical analysis. KY and NN participated in the design of the study and supervised the above researchers/clinicians and prepared the manuscript. All authors read and approved the final manuscript.

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Searching for Genes for Cleft Lip and/or Palate Based on Breakpoint Analysis of a Balanced Translocation t(9;17)(q32;q12)

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Objective: **Identification of the breakpoints of disease-associated chromosome rearrangements can provide informative clues to a positional cloning approach for genes responsible for inherited diseases. Recently, we found a three-generation Japanese family segregating balanced chromosome translocation t(9;17)(q32;q12). One of the subjects had cleft lip and palate. We examined whether regions near the breakpoint could be associated with cleft lip and/or palate.**

Methods: **We determined the breakpoints involved in the translocation by fluorescence *in situ* hybridization analysis and subsequent long-range polymerase chain reaction. In order to study the role of these disrupted regions in nonsyndromic cleft lip and/or palate, we performed mutation analysis and a haplotype-based transmission disequilibrium test using tagging single-nucleotide polymorphisms in the flanking regions of the breakpoints in white and Filipino nonsyndromic cleft lip and/or palate populations.**

Results: **Sequence analysis demonstrated that two genes, *SLC31A1* (solute carrier family 31 member 1) on chromosome 9 and *CCL2* (chemokine ligand 2) on chromosome 17, were rearranged with the breaks occurring within their introns. It is interesting that *SLC31A1* lies closed to *BSPRY* (B-box and SPRY domain), which is a candidate for involvement with cleft lip and/or palate. Some of the variants in *BSPRY* and *CCL2* showed significant *p* values in the cleft lip and/or palate population compared with the control population. There was also statistically significant evidence of transmission distortion for haplotypes on both chromosomes 9 and 17.**

Conclusions: **The data support previous reports that genes on chromosomal regions of 9q and 17q play an important role in facial development.**

KEY WORDS: *association analysis, balanced chromosomal translocation,* BSPRY, CCL2, *cleft lip and palate, haplotype, HapMap,* SLC31A1

Nonsyndromic cleft lip and/or palate (CL/P) is a common congenital anomaly of complex etiology, with the birth prevalence being approximately 1 per 700 live births (Mossey and Little, 2002; Murray, 2002). Previous family and population studies indicate that both genetic and environmental factors are involved in the occurrence of this malformation. Prior sequencing analysis of CL/P has indicated roles for mutations in *MSX1*, *FOXE1*, *GLI2*, *MSX2*, *SKI*, *SPRY2*, *RYK*, *FGF1*, *FGF2*, and *FGF8* in the etiology of the birth defect (Ichikawa et al., 2006; Jezewski et al., 2003; Riley et al., 2007; Vieira et al., 2005). In addition, polymorphisms in *IRF6* have been found to be associated with CL/P (Blanton et al., 2005; Ghassibe et al., 2005; Park et al., 2007; Scapoli et al., 2005; Zucchero et al., 2004).

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Because clefts are complex and caused by multiple interacting genes, we do not expect that single gene disruptions in all individuals with a chromosomal break will manifest as CL/P. However, in some cases, identification of the breakpoints of disease-associated chromosome translocations can be an effective way to identify genes and/or chromosomal regions contributing to the occurrence of CL/P. The positional cloning approach for disease-associated genes using chromosome rearrangements has successfully identified four relevant genes (*CLPTM1* [Yoshiura et al., 1998], *SATB2* [FitzPatrick et al., 2003], *SUMO1* [Alkuraya et al., 2006], and *FGFR1* [Kim et al., 2005]) as candidates for CL/P. These findings suggest that identification of the genes disrupted in a cleft/translocation case can give us insight into some of the pathways involved in this birth defect and provide candidates for analysis in cases independent of the index family.

Recently, we identified a Japanese family segregating a balanced chromosome translocation t(9;17)(q32;q12). Of three generations we examined, one individual is affected with cleft lip and palate. Genome-wide linkage studies have suggested that the region 9q21 has significant linkage with CL/P (Marazita et al., 2004). Therefore, genes in this region of chromosome 9 may have an important role in facial development. In this study, we identified the breakpoints of t(9;17)(q32;q12) in the patient by fluorescence *in situ* hybridization (FISH) analysis and identified candidate genes by polymerase chain reaction (PCR) and DNA sequencing. We then carried out a mutation search and a case-control association study of candidate genes. In addition, extended single-nucleotide polymorphism (SNP) analysis of the chromosomal regions adjacent to the translocation breakpoint was performed.

Materials and Methods

Clinical Report

The proband was a male infant born prematurely by cesarean section at 24 weeks gestation after an uneventful pregnancy. The maternal age was 25 years and paternal age 26 years at the time of his birth. The parents were nonconsanguineous, and there was no family history of malformations. His birth weight and height were 706 g and 31.8 cm, respectively (10th centile = 560 g, 90th centile = 774 g). The patient was in normal development at 24 weeks gestation. The circumferences of his head and chest were 23 cm and 21 cm at birth, respectively. He had apparent ocular hypertelorism, a bilateral cleft lip and palate, and a mild nasal flattening. Echocardiography showed a small atrial septal defect, which had closed by 2 years of age. At 6 years after birth, his weight and height were normal for age at 20.1 kg and 114.6 cm, respectively. He shows no evidence of developmental delay. Routine chromosome-banding analysis of the patient revealed an apparently balanced translocation between the long arm of chromosome 9 and chromosome 17: 46,XY, t(9;17)(q32;q12). His father and paternal grandmother had the same translocation seen in the patient; whereas, his mother had a normal karyotype (Fig. 1). Neither of the other translocation carriers had a cleft or malformations, and there was no family history of CL/P.

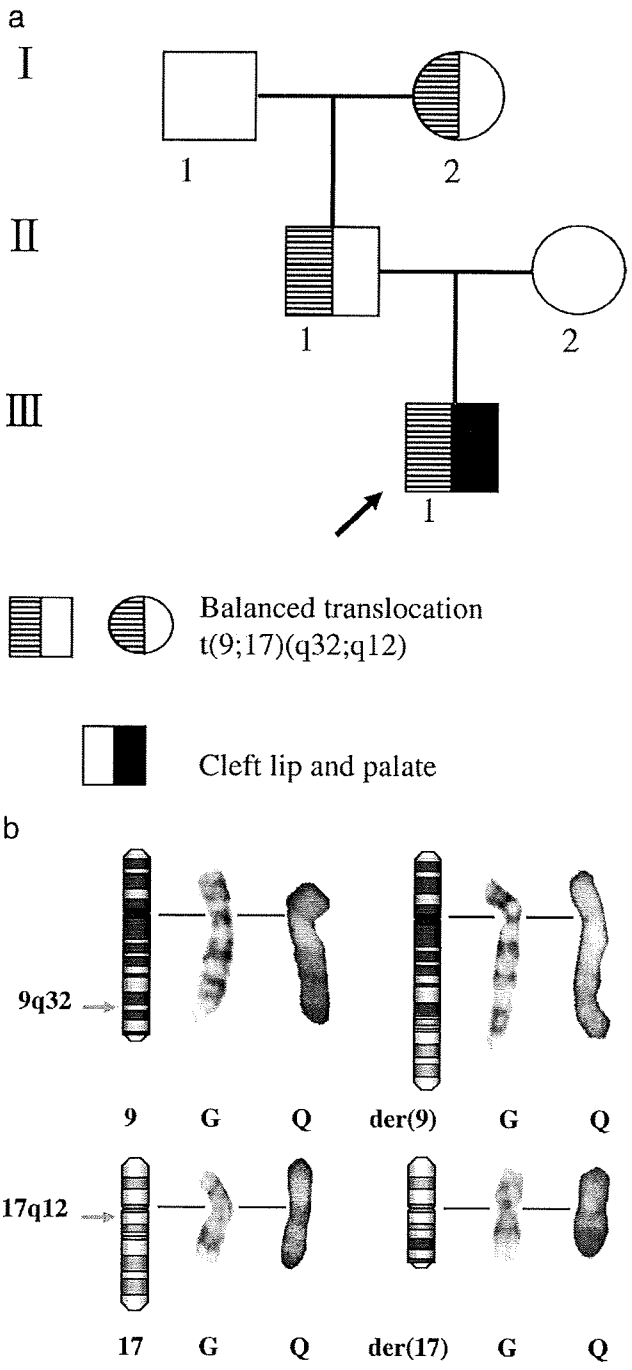

FIGURE 1 Case presentation. a: Pedigree of the family examined. b: Chromosomal breakpoints in the patient (III-1). Ideogram and a partial karyotype are shown. Chromosomal breakpoints are indicated by arrows.

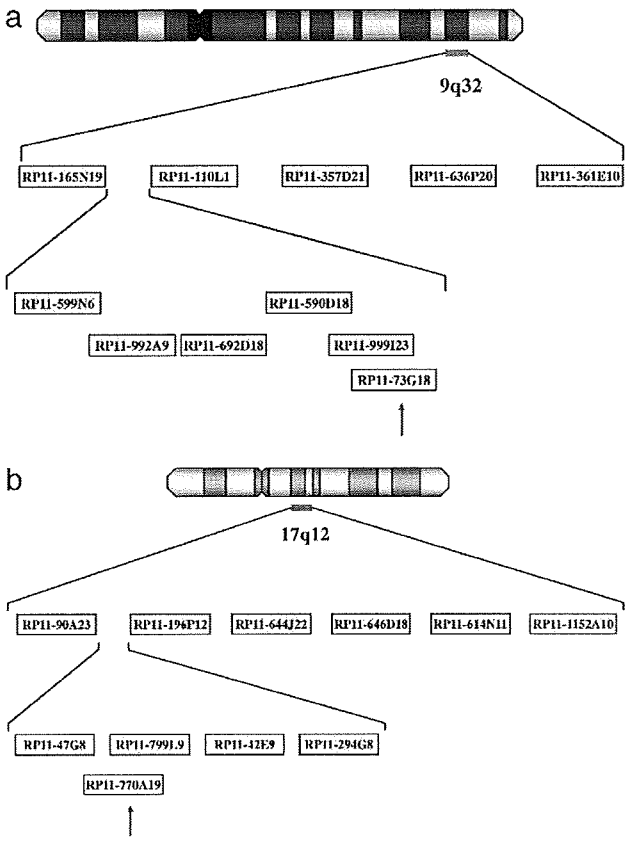

FIGURE 2 BAC clones used for FISH analysis. a: A physical map of BAC clones covering the 9q32 subchromosomal band. b: A physical map of BAC clones covering the 17q12 subchromosomal band.

Identification of Translocation Breakpoint

Fluorescence In Situ *Hybridization Analysis*

Breakpoint mapping of the translocation in the patient was initiated by FISH analysis, according to published procedures (Ono et al., 1997). Bacterial artificial chromosome (BAC) clones were identified using the genome maps provided by the National Center for Biotechnology Information (http://www.ncbi.nlm.org) and University of California, Santa Cruz (UCSC) (http://genome.cse.ucsc.edu) genome browsers. Figure 2 shows the BAC clones on chromosomes 9 and 17 used in the present study.

Cloning of the Translocation Breakpoint

We carried out a series of long-range PCR amplifications of DNA fragments to detect the recombinant sequence caused by the translocation. Primer sequences were designed based on the FISH results, with one forward primer on chromosome 9 and one reverse primer on chromosome 17, and were evaluated by RepeatMasker (http://www.repeatmasker.org/) to avoid nonspecific amplification of repetitive sequence. The PCR products then were subcloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA). Additional nested primers were designed to obtain a DNA fragment including the breakpoint on der(9) using the subcloned plasmid DNA from the patient and genomic DNA of members of his family.

DNA Sequencing

The PCR products were labeled with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit according to the manufacturer's instruction (Applied Biosystems, Carlsbad, CA). The products were purified, and then analyzed on an ABI 3700 automated sequencer (Applied Biosystems). The Applied Biosystems sequence software (version 2.1.2) was used for lane tracking. Chromatograms were transferred to a UNIX workstation, base-called with PHRED 4.0 (http://droog.mbt.washington.edu/poly_doc40.html) and results viewed with the CONSED program (version 4.0) (Nickerson et al., 1997). The UCSC and the Ensembl databases (http://www.ensembl.org) were used to detect potential genes around the breakpoint.

Mutation Search of Candidate Genes in Case/Control CL/P Population

To examine the association of the disrupted genes with the occurrence of CL/P in individuals unrelated to this family, we screened for mutations of the untranslated regions (UTR), exons and exon-intron boundaries of the two rearranged genes on chromosome 9 and chromosome 17. In addition, any plausible candidate gene adjacent to the breakpoint was screened. We sequenced 90 individuals affected with isolated CL/P from the Philippines and 90 from Iowa. The control group comprised 90 samples from unrelated Filipinos and whites provided by Centre d'Etudes du Polymorphisme Humaine (Dausset et al., 1990). Standard chi-square tests of association were used to compare the frequencies of each variant found by sequencing between groups of patients and controls.

Haplotype Analysis Adjacent to the Translocation Breakpoint

To determine whether the chromosomal regions adjacent to the breakpoint could play a role in the development of CL/P, we carried out the haplotype-based transmission disequilibrium test. Four tagging SNP markers (rs974230, rs1330691, rs4596714, rs3750534) adjacent to the breakpoint on chromosome 9 and three markers (rs16561, rs725276, rs1029719) on chromosome 17 were selected, based on the HapMap (http://www.hapmap.org/index.html.en) database. The TaqMan genotyping for these SNPs was performed with the ABI PRISM 7900 Sequence Detection System (Applied Biosystems) using 371 case/parent families from the Philippines and 206 from Iowa. Haplotype-based transmission disequilibrium statistics