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A SNP in the *ABCC11* gene is the determinant of human earwax type

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Human earwax consists of wet and dry types. Dry earwax is frequent in East Asians, whereas wet earwax is common in other populations. Here we show that a SNP, 538G→A (rs17822931), in the *ABCC11* gene is responsible for determination of earwax type. The AA genotype corresponds to dry earwax, and GA and GG to wet type. A 27-bp deletion in *ABCC11* exon 29 was also found in a few individuals of Asian ancestry. A functional assay demonstrated that cells with allele A show a lower excretory activity for cGMP than those with allele G. The allele A frequency shows a north-south and east-west downward geographical gradient; worldwide, it is highest in Chinese and Koreans, and a common dry-type haplotype is retained among various ethnic populations. These suggest that the allele A arose in northeast Asia and thereafter spread through the world. The 538G→A SNP is the first example of DNA polymorphism determining a visible genetic trait.

Earwax (cerumen) is a secretory product of ceruminous apocrine glands. Human earwax is a mendelian trait consisting of wet and dry types[1]. The wet earwax is brownish and sticky, whereas the dry type lacks cerumen. The wet cerumen phenotype is completely dominant to the dry type. The dry type is seen frequently (80–95%) among East Asians[2–4], but uncommon (0–3%) in populations of European and African origins. Intermediate frequencies (30–50%) of the dry type are seen in populations of Southern Asia, the Pacific Islands, Central Asia and Asia Minor, as well as among the Native North American and Inuit of Asian ancestry[2,5]. These figures show geographical gradient distributions. Earwax type may play a role in the axillary odor and possibly in breast cancer susceptibility[6], although the association with breast cancer remains controversial[7]. Using a linkage analysis, we have previously assigned the earwax gene locus to the pericentromeric region of chromosome 16 (ref. 8).

With the aim of further mapping of the earwax locus, we performed genotyping and case-control study of 64 Japanese individuals with dry earwax and 54 with the wet type using 134 CA repeat markers. Although 12 markers showed an association with a P value of $\log_{10} < -2.3$, they did not allow finer mapping because their distribution was too wide. Nevertheless, six of them were located to a ~2.5-Mb pericentromeric region of 16q. Another association study using 36 SNPs of the samples showed that two SNPs (B81540.1 and IMS-JST141676) at this region gave P values of 4.0×10^{-9} and 7.6×10^{-6}, respectively. All 64 individuals with dry earwax were

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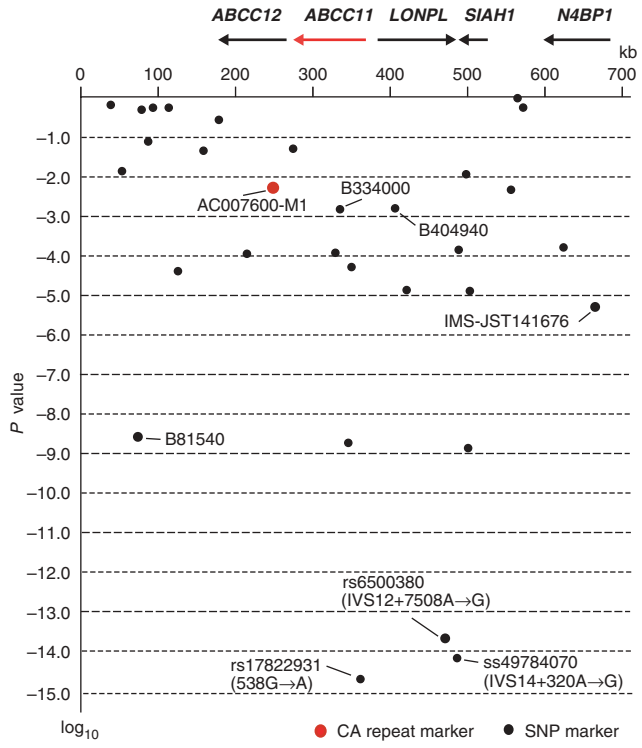

Figure 1 Four genes, *ABCC12*, *ABCC11*, *LONPL* and *SIAH1*, and 31 polymorphic DNA markers located in the ~600-kb region between B81540 and IMS-JST141676. Three intragenic polymorphisms, rs17822931 (538G→A) in *ABCC11*, rs6500380 (IVS12+7508A→G) and ss49784070 (IVS14+320A→G) in *LONPL*, gave a *P* value of $<2.0 \times 10^{-14}$ in an association study of 118 volunteers (64 dry-type and 54 wet-type) whose DNA was collected in anonymous manner and their earwax type self-declared, and were in strong linkage disequilibrium ($r^2 \approx 1.00$) in the Japanese population. Further genotyping in another series of 126 Japanese individuals (88 with dry earwax and 38 with wet earwax) whose earwax types were clinically confirmed showed that rs17822931 is the determinant of earwax type.

homozygotes for one allele each, consistent with an autosomal recessive trait and supporting the presence of the earwax locus in this region. According to GenBank, the two-SNP interval is approximately 600 kb and contains five genes: *ABCC12*, *ABCC11*, *LONPL*, *SIAH1* and *N4BP1*. We performed an association study of the 118 samples using 37 SNPs within the five-gene interval and found that rs17822931 (538G→A, G180R) in *ABCC11* exon 4, rs6500380 (IVS12+7508A→G) in *LONPL* intron 12 and ss49784070 (IVS14+320A→G) within an Alu-repetitive sequence in *LONPL* intron 14 showed the lowest *P* values ($<2.0 \times 10^{-14}$; **Fig. 1**). Genotyping at the three SNP loci showed that 63 of 64 samples from individuals with the dry type were AA-AA-AA and one GA-GA-GA; of the samples from individuals with wet earwax, 6 of the 54 were GG-GG-GG, 33 were GA-GA-GA and 15 were AA-AA-AA (**Supplementary Table 1** online). We performed LD analysis for the 37 SNPs and found two LD blocks among the 118 Japanese: one large block containing the 3′-region of *PHKB*, the neighboring gene ~450 kb centromeric to *ABCC12*, and another block including the three SNPs as well as *SIAH1* (**Fig. 2**). The three SNPs were in strong LD ($r^2 \approx 1.0$) in the Japanese, and only two haplotypes, G-G-G and A-A-A, were predicted in the Japanese in our samples. Although these findings suggest that one of the three SNPs defines earwax type, some samples showed phenotype-genotype inconsistency.

Genotyping of a new series of 126 Japanese individuals whose earwax types were identified otologically showed that 87 of 88 individuals with dry earwax were AA homozygotes, and all 38 individuals with the wet type were either GA heterozygotes or GG homozygotes at each of the three SNP loci (**Table 1** and **Supplementary Table 1** online). Among the SNPs, only rs17822931 is nonsynonymous (G180R); rs6500380 does not create any splicing sites nor affect splicing factor binding motifs or known promoter sequences, and ss49784070 is located within the Alu-repetitive sequence, all supporting rs17822931 as an earwax determinant. One exceptional individual showed discordance between phenotype (dry earwax) and genotype (GA heterozygous for each of SNP); sequencing showed that he had a 27-bp deletion (3939–3965del27, or Δ27) in *ABCC11* exon 29 (the exon numbering is according to a previous report[9]) downstream of the G/A site of the gene. The Δ27 was found in his G allele in *ABCC11* (**Fig. 3**). These may support an idea of loss-of-function of the allele G, leading to the dry phenotype. Thus, the frequency of the dry earwax phenotype among residents of Nagasaki, Japan is 0.698 (88/126), and that of allele A at rs17822931 is 0.829 (206/252).

We genotyped a total of 33 different populations around the world at rs17822931. East Asians have high frequencies of allele A. The highest value (100%) was observed in Northern Han Chinese and Koreans, and the second highest in Mongolians, Chinese from other areas of China and mainland Japanese. Africans from various sub-Saharan nations and African Americans showed the lowest frequency. Intermediate frequencies were observed in other populations (**Table 1** and **Supplementary Table 2** online). A world map of the frequency of allele A shows a north-south downward gradient from Northern China toward Japan and Southern Asia as well as an east-west gradient from Siberia toward Europe (**Fig. 4**), similar to that found previously for earwax phenotype[2–5]. We also genotyped for Δ27 in various populations. Notably, it was found in 2 of 20 native North Americans, 1 of 10 Andeans, 9 of 30 Bolivians and 1 of 50 Thai individuals, in addition to 1 of 334 Japanese individuals, but none in other populations. All 14 individuals were heterozygous for Δ27. As Δ27 seems independent of genotype at rs17822931, it is a deletion polymorphism that is relatively frequent in subsets of the Native Americans. However, as the deletion among Bolivians was located in an LD block next to the block involving rs17822931 (**Fig. 2c**), it remains to be investigated whether Δ27 has a single origin. Analysis of an rs17822931-rs6500380-ss49784070 haplotype structure showed a haplotype G-G-G in all ten African-Americans and in Japanese with wet earwax, suggesting that it is the ancestral state. In Chinese individuals, Native Americans, Bolivians and the CEPH family members, the calculated r^2 value between rs6500380 and ss49784070 was 1.00, whereas the values between rs17822931 and rs6500380 in these populations and Southern Chinese were 0.82, 0.58, 0.62 and 0.67, respectively. In all populations examined but the CEPH families, A-A-A was the major haplotype with variable frequencies (0.47–0.90), whereas G-G-G was the major haplotype in the CEPH families (**Supplementary Table 3** online). We constructed LD blocks, which showed almost the same structure among the Japanese, Native Americans, Bolivians and Chinese (**Fig. 2**). Several recombinations between rs17822931 and rs6500380 were evident in all ethnic groups in which haplotypes were analyzed. Two other SNPs (B334000 and B404940) gave *P* values of 1.3×10^{-3} and 1.5×10^{-3} in an association study of 87 Japanese individuals with dry earwax for respective minor alleles. In addition, a CA repeat marker (AC007600M1) in intron 21 of *ABCC12* (**Fig. 1**) did not show

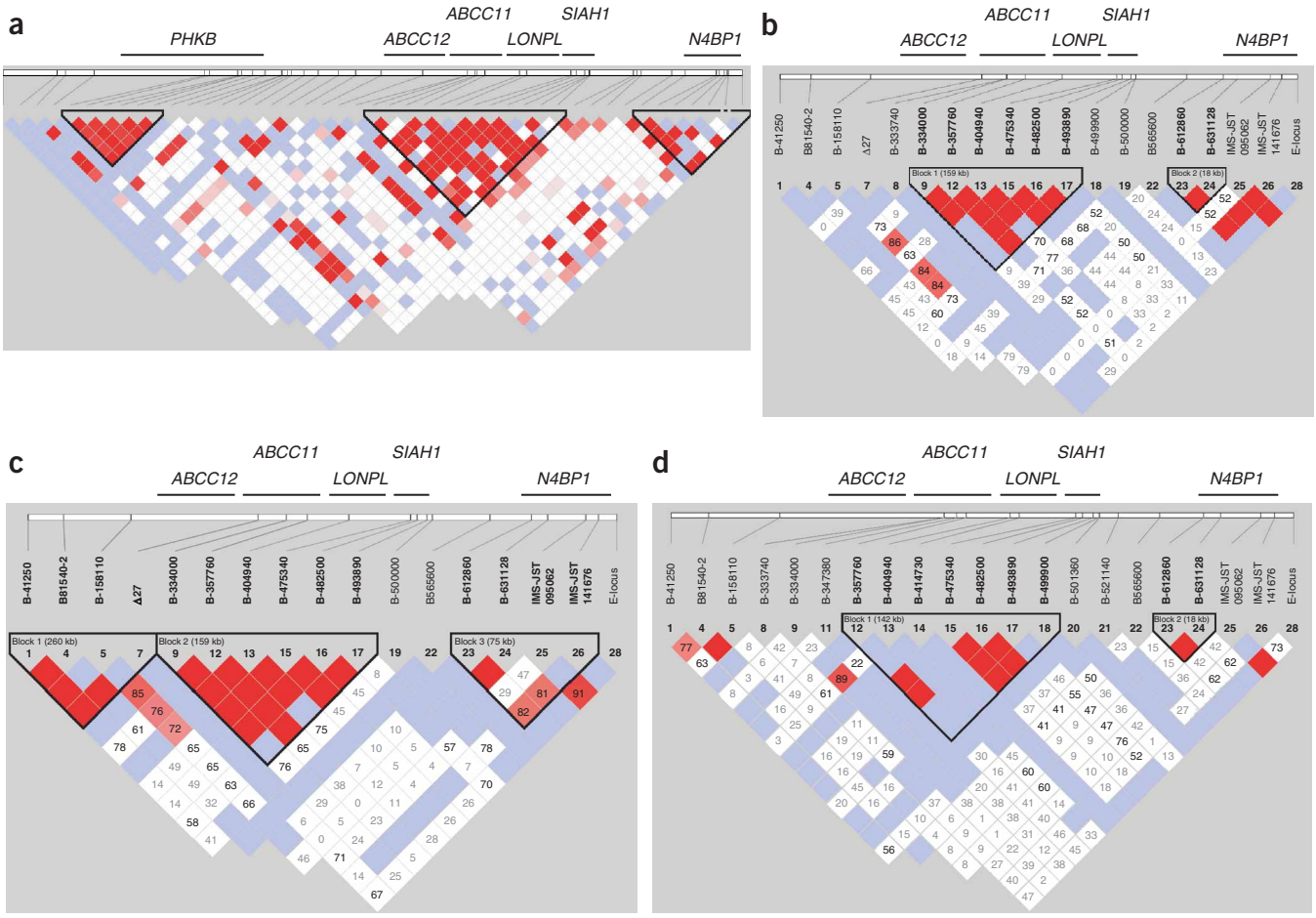

Figure 2 Linkage disequilibrium (LD) analysis of candidate region. Three LD blocks were identified in the Japanese. (**a**) One block at the most centromeric region includes the *PHKB* gene, another at the middle contains *ABCC12*, *ABCC11*, *LONPL* and *SIAH1* and the other at the most telomeric region includes *N4BP1*. A haplotype block including rs17822931, rs6500380 and ss49784070 was observed in Native Americans (**b**), Native Bolivians (**c**) and Southern Han Chinese (**d**). Because of few informative SNPs in the Chinese, we could not define any clear LD blocks other than the block constructed by the three SNP sites. Also, we did not observe any LD blocks in African-American by the SNPs we used. SNPs used for haplotype analyses are listed in **Supplementary Table 4**.

homozygosity among Japanese individuals with dry earwax. Thus, some recombinations must have occurred even in the Japanese, although we did not detect any recombination among rs17822931, rs6500380 and ss49784070. Thus, the high frequency of the dry-earwax haplotype A-A-A with little recombination suggests that it has risen rapidly in frequency from a recent founder. Rare mutation(s) at other site(s) such as Δ27 in *ABCC11* also contribute to dry earwax formation in a subset of individuals.

We compared the cGMP transport activity of two LLC-PK1 cell lines expressing human *ABCC11*, one carrying allele G (LLC-PK1-G) and another carrying allele A (LLC-PK1-A). *ABCC11* encodes the multidrug resistance–associated protein 8 (MRP8) that consists of 1,382 amino acids and contains two ATP-binding domains and 12 transmembrane domains[9–11]. LLC-PK1-A cells expressed *ABCC11* mRNA three times more abundantly than LLC-PK1-G (**Fig. 5a**). ATP-dependent cGMP transport activity of plasma membrane vesicles prepared from LLC-PK1-G cells expressing the Gly180 type of MRP8 was significantly higher than the activity of cells expressing the Arg180 type, as measured in cGMP concentrations ranging from 100 μM to 500 μM (**Fig. 5b**). On the other hand, transport activity of the membrane expressing MRP8 with Arg180 was similar to that of mock vector-transfected LLC-PK1 cells.

The G180R substitution in rs17822931 (538G→A) is located at the first transmembrane domain of MRP8. In addition, Δ27 in exon 29 was found on allele G in a G/A-heterozygous individual with dry earwax. These may lead to loss of function of the protein and indeed strengthen the evidence that *ABCC11* has a role in determining the human earwax trait. A recent study demonstrated that MRP8 is an amphipathic anion transporter and functions as a drug efflux pump for purine and pyrimidine nucleotide analogs such as cAMP and cGMP[12]. Because MRP8 can transport a variety of lipophiles[13], and earwax contains various aliphatic compounds, aromatic hydrocarbons and other molecules[14], it is reasonable that wild-type MRP8 functions to secrete earwax. Histological findings of the wet-type ceruminous gland containing a 'light granule' that might be a secretory vesicle[15] also support the role of *ABCC11*. Individuals with fatal surfactant deficiency (that is, a genetic disorder caused by mutations in *ABCA3*, one of the ABC transporter genes) lack surfactant in their alveolar type II cells[16]. Similarly, as no light granules and microvilli on the cell surface are observed in the dry-type

Table 1 Genotypes at the rs17822931 site and frequencies of alleles A and Δ27 of *ABCC11* among different ethnic populations

Ethnic populations	Tribes or inhabitants studied	No. of individuals with genotypes: AA	(frequency)	GA	GG	No. of individuals genotyped	Frequency of allele A	Frequency of allele Δ27	Collected by
Korean	Taegu inhabitant	99	(1.000)	0	0	99	1.000	0/190	D.-K.K.
Chinese	Shanxi inhabitant (Northern Han Chinese)	74	(1.000)	0	0	74	1.000		N.S.
	Jingsu inhabitant (Northern Han Chinese)	110	(0.924)	9	0	119	0.962		N.S.
	Inhabitants of Fujian, Guanjgdong and Hunan Provinces (Southern Han Chinese)	249	(0.750)	63	8	332	0.845		N.S.
	Han Chinese from multi-regions	42	(0.808)	10	0	52	0.904	0/104	D.S.L.
Mongolian	Khalkha tribe	126	(0.759)	36	4	166	0.867	0/252	T.I., A.G.
Japanese	Nagasaki inhabitant (western prefecture of Japan mainland)	87	(0.690)	35[a]	4	126	0.829	1/668	N.N.
	Okinawa people (southwestern prefecture of Japan)	30	(0.517)	25	3	58	0.733		T.K.
	Yonakuni islander (western island of Japan)	13	(0.433)	15	2	30	0.683	0/60	S.S.
	Ainu in Biratori-Nibutani village in Hokkaido	32	(0.552)	24	2	58	0.759	0/116	T.I., N.N.
Vietnamese	People from multi-regions	82	(0.536)	60	11	153	0.732		N.Na.
Dravidian	Inhabitants of Tamil Nadu in India	27	(0.540)	17	6	50	0.710	0/100	T.I.
Thai	Northern Thai (Lahu, Shan, Lisu, Hmong, Akha, Mlabri and Karen (Mae-sot Thai) tribes combined)	215	(0.505)	163	48	426	0.696	0/158 (Karen)	T.I., K.H.
	Central Thai in Bangkok	31	(0.633)	10	8	49	0.735	1/100 (Bangkokian)	T.I.
	Southern Thai (Urak Lawoi and Sakai tribes combined)	2	(0.026)	23	52	77	0.175	0/52 (Sakai)	T.I.
Vedda	Native people in Sri Lanka	7	(0.350)	12	1	20	0.650		T.I.
Indonesian	Dayak tribe in Kalimantan	12	(0.293)	23	6	41	0.573		T.I.
	Toraja and Bugis tribes in Sulawesi	27	(0.270)	49	24	100	0.515		T.I.
	Floresian	18	(0.300)	25	17	60	0.508		T.I.
	Sumbanese	9	(0.180)	16	25	50	0.340		T.I.
	Dani tribe in Irian Jaya	0	(0.000)	2	31	33	0.030		T.I.
Malaysian	Sabah in North Borneo	24	(0.393)	27	10	61	0.566	0/132 (Sabah)	K.H.
	Bentong tribe	8	(0.113)	40	23	71	0.394	0/138	K.H.
Taiwanese	Taiwan Aborigine (Yami and Ami combined)	34	(0.330)	48	21	103	0.563	0/100 (Ami)	T.I.
Native American		6	(0.300)	8	6	20	0.500	2/40	R.K.
Philippino	Palawan	11	(0.229)	23	14	48	0.469		T.I.
Easter Islander		4	(0.138)	18	7	29	0.448		S.S.
Bolivian	Aymara inhabitants	5	(0.167)	14	11	30	0.400	9/60	S.S.
Kazakh		6	(0.200)	11	13	30	0.383		G.K.A.
Native Paraguayan	Ayoreos	2	(0.040)	34	14	50	0.380	0/98	K.H.
	Sanapana	0	(0.000)	14	61	75	0.093	0/150	K.H.
Russian		5	(0.045)	45	62	112	0.246	0/208	V.A.S.
Solomon Islander		2	(0.323)	25	35	62	0.234	0/122	K.H.
Pacific islander		1	(0.143)	1	5	7	0.214	0/14	Coriell
French	From the CEPH families	1	(0.083)	3	8	12	0.208	0/24	CEPH
Andean people		1	(0.100)	2	7	10	0.200	1/20	Coriell
Hungarian		0	(0.000)	4	6	10	0.200	0/20	Coriell
Jewish	Ashkenazi	0	(0.000)	4	6	10	0.200	0/20	Coriell
Ukrainian		0	(0.000)	15	27	42	0.179	0/84	V.A.S.
Papuan	Papua, New Guinea	1	(0.026)	11	26	38	0.171	0/68	T.I.
European American	From CEPH families without the French and Venezuelans	1	(0.012)	16	65	82	0.110	0/164	CEPH
Vanuatu islander	Aneityum and Santo islanders combined	1	(0.011)	17	74	92	0.103	0/266 (Any and Gau islanders)	K.H.
Iberian		0	(0.000)	2	8	10	0.100		Coriell
Colombian		0	(0.000)	2	15	17	0.059	0/34	S.S.
Venezuelan	Inhabitants of Ye'Kuana and Sanuma villages	0	(0.000)	3	29	32	0.047	0/64	S.S.,CEPH
African	From various sub-Saharan nations	0	(0.000)	1	10	11	0.045	0/22	C.K.M.
African American		0	(0.000)	0	10	10	0.000	0/20	Coriell

D.-K.K., D.-K. Kim; N.S., N. Saitou; N.N., N. Niikawa; D.-S.L., D.-S. Liang; T.I., T. Ishida; A.G., A. Garidkhuu; T.K., T. Kaname; S.S., S. Sonoda; N.Na., N. Natsume; K.H., K. Hirayama; R.K., R. Komaki; G.K.A., G.K. Alipov; V.A.S., V.A. Saenko; C.K.M., C.K. Mapendanno; Coriell, from the Coriell Institute; CEPH, from the CEPH families.
[a]One exceptional case of dry cerumen who has Δ27 on the G allele is included.

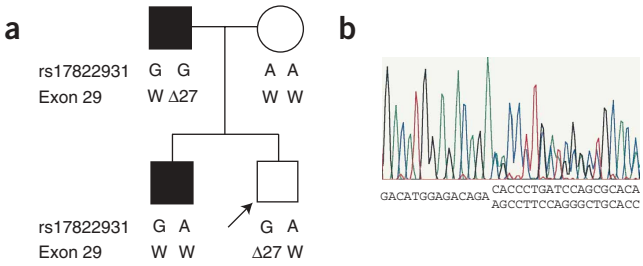

Figure 3 Pedigree analysis of an individual showing phenotype-genotype discrepancy (at rs17822931). (**a**) Family tree and genotypes at rs17822931 and exon 29 in *ABCC11*. Filled symbols: wet earwax; open symbols, dry earwax. Family analysis clearly indicates that allele Δ27 found in the proband with dry cerumen (arrow) was transmitted from his father and shows that alleles G and Δ27 are on the same chromosome and compose a haplotype. (**b**) Electropherogram of exon 29 from the proband. A 27-bp intraexonic deletion is evident in the proband.

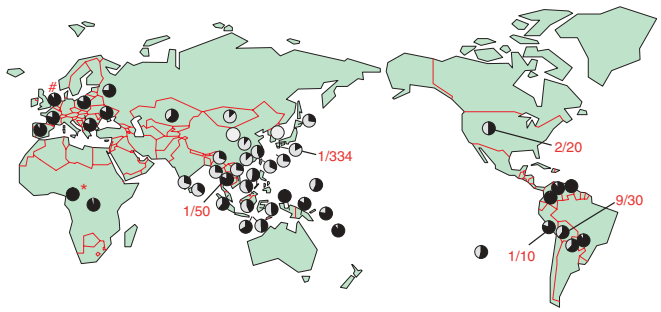

Figure 4 Worldwide frequency of allele A (open portion in each circle). There is a north-south downward gradient from Northern China toward Southern Asia with a peak in East Asians, and an east-west downward gradient from Siberia toward Europe. Another mutation, a 27-bp deletion (Δ27) in exon 29 of *ABCC11*, independent of rs17822931 (538G→A), was also observed in subsets of individuals of Asian ancestry. Red numbers indicate individuals with Δ27; #, US citizens of Northwest European ancestry (from the CEPH families); *, US citizens of sub-Saharan African ancestry (from the Coriell Institute for Medical Research).

ceruminal gland[15], earwax may be relevant to the surfactant production and secretion process.

It is believed that ancient Northeast Eurasians had reached a high frequency of dry earwax, and the population thereafter expanded in size, showing geographic range among East Asians[2]. Although our data showing gradient distributions of allele A with a peak in North China and Korea favor that hypothesis, its origin should be verified by mutational diversity analysis on the haplotype carrying allele A. An independent origin of the same mutation (538G→A) is unlikely, because Southern Chinese individuals, Japanese individuals, Native Americans, the CEPH families and Native Bolivians with dry earwax all share the haplotype carrying allele A. Therefore, the geographical cline of dry earwax may reflect migration of ancient Northeast Eurasians. This expansion of the dry type may be a result of a certain selective advantage of the dry cerumen[2]. The genomic region containing four genes, *ABCC12*, *ABCC11*, *LONPL* and *SIAH1*, is indeed a SNP desert that may reflect such strong directional selection important for the modern human evolution[17]. Furthermore, according to the International HapMap Project, rs17822931 is one of 32 high-differentiation, nonsynonymous SNPs that may have experienced geographically restricted selection pressures[18]. The high frequency of allele A in *ABCC11* among Native Americans may reflect their ancestors' migratory wave(s) from Siberia over the Bering land bridge to the American continent[19–21]. In this context, Δ27 found in subsets of Native North and South Americans, in addition to one subset each of Japanese and Thai individuals, is of great interest. However, where and when it originated remains unknown.

The phenotypic implications of earwax remain unknown. Insect trapping, self-cleaning and prevention of dryness of the external auditory canal are its plausible functions[2]. The axillary odor is known to be associated with wet-type cerumen and intensified by sweating. Histological study of the axillary skin from a patient with bromhidrosis showed numerous and large-sized apocrine glands[22]. In nonhuman mammals, secretory products of apocrine sweat glands such as body odor may have a role in sexual attraction as a sort of pheromone. According to the genome sequence database and a PCR search by us, the chimpanzee, orangutan, white-handed gibbon, Japanese macaque and the dog have the orthologue of *ABCC11*, but mice and rats lack it[23]. Instead, urinary odor types determined by the major histocompatibility complex (MHC) have a similar biological role in the rodents[24]. As ancient Northeast Eurasians are believed to have lived in a cold environment, they might have acquired some selective advantages, such as less axillary odor or sweating, as an adaptation to the cold climate.

METHODS

SNP identification, genotyping, case-control study and haplotyping. Study protocols were approved by the Institutional Review Boards of each institution joining the study. Genomic DNA was extracted from whole blood of volunteers using the standard method or from their fingernails with ISOHAIR (Nippon Gene). Volunteers gave written informed consent. Other DNA samples were purchased from the Coriell Institute for Medical Research. We searched for CA repeat sequences and SNPs around the ~5.9-cM segment containing *D16S3093* and *D16S311* and withdrew coding SNP (cSNP) data from the JSNP database because the earwax type is a single-gene trait, and the JSNP data are constructed from the Japanese, in which the dry type is known be the major

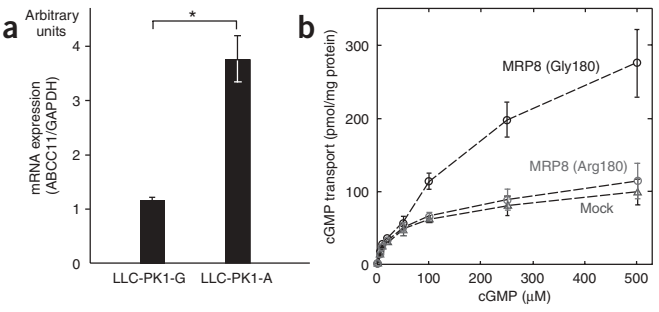

Figure 5 *ABCC11* expression and cGMP transport in LLC-PK1 cells transfected with allele A and those with allele G. (**a**) Expression of *ABCC11* mRNA was compared with *GAPDH* expression from same cells, using quantitative PCR. Results from three independent experiments are shown (mean ± s.e.m.). LLC-PK1-A cells expressed *ABCC11* three times more abundantly (3.24 ± 0.64) than LLC-PK1-G (*$P < 0.001$, Student's *t*-test). (**b**) ATP-dependent transport of cGMP in plasma membrane vesicles prepared from *ABCC11*-expressing LLC-PK1 cells stably expressing Arg180 and Gly180 types of MRP8 and from mock-transfected LLC PK1 cells (mean ± s.d.). K_m values calculated for cGMP were 7.8 μM and 302 μM, representing MRP8 (Arg180)/mock and MRP8 (Gly180)-mediated transport of cGMP, respectively.

phenotype[2]. As we had previously found a shortage of SNPs in this region[17,18], we identified 15 new SNPs by sequencing the region among eight individuals with wet earwax. A total of 134 CA repeat polymorphisms from the region and 15 new SNPs (three already deposited in dbSNP) as well as 21 registered SNPs were used as markers for the first-step case-control studies by genotyping 118 Japanese volunteers (64 individuals with dry earwax and 54 controls with wet earwax) whose DNA samples were collected in an anonymous manner and whose earwax types were self-declared. We found two SNPs, B81540.1 and IMS-JST141676, that were homozygous in 64 individuals with dry earwax. This led us to focus on a ~600-kb region between the two SNPs, especially on a four-gene (*ABCC12*, *ABCC11*, *LONPL* and *SIAH1*) interval, on which we performed PCR-based genome sequencing of genomic DNA except on long repetitive elements in the eight wet-type individuals. If a given SNP within a gene is close to the earwax locus, these individuals are expected to show 80–90% heterozygosity, in view of the previously reported earwax phenotype data in the Japanese[2,4]. In this interval, we found 37 SNPs (15 registered and 22 novel SNPs) and used them for another association study among the same 118 samples above and for the construction of LD blocks to confirm the 600-kb region as a candidate region. LD blocks were constructed using the Haploview program[25]. Because none of the SNPs above completely explained the earwax phenotype as a mendelian trait, we collected blood from another series of 126 Japanese volunteers (88 dry-type and 38 wet-type individuals) from Nagasaki City, whose earwax types were identified by a medical practitioner, and used them for the second-step association study.

Using the Haploview program[25], we constructed haplotypes including 24 SNPs and Δ27 in the 600-kb interval and its flanking region in Native Americans, Native Bolivians and Southern Han Chinese individuals. Primer sequences for construction of haplotypes in various populations are listed in **Supplementary Table 4** online.

Complete genome sequencing of the individuals with dry earwax and wet earwax. We adopted two strategies, PCR-based complete genome sequencing in eight wet-type individuals and BAC clone–based sequencing in a wet/dry-type heterozygote for the genomic regions that could not be amplified from the eight samples. Comparisons between the PCR-based sequence and the complete contig sequence, and between two BAC clones containing the allele responsible for the wet- or dry-type phenotype showed no structural abnormality or any base changes other than SNPs found among the eight wet-type individuals. The complete sequence consisted of 341,290 bp and contained four genes (*ABCC12*, *ABCC11*, *LONPL* and *SIAH1*) spanning an LD block.

LLC-PK1 cell lines expressing allele A or G of human *ABCC11*, preparation of plasma membrane vesicles from these cells and detection of ATP-dependent transport of cGMP. Full-length cDNA carrying allele A (dry-type allele) and that carrying allele G (wet-type allele) of human *ABCC11* were inserted into pcDNA3.1-Hygro (Invitrogen) and transfected into pig-derived LLC-PK1 cells (Health Science Research Resources Bank (HSRRB) cell bank) using Lipofectin reagent (Invitrogen). Total RNA was prepared from *ABCC11*-expressing cell lines (LLC-PK1-A and LLC-PK1-G carrying alleles A and G, respectively) and was used for reverse transcription (RT). Real-time PCR quantification was carried out using a 7900HT Sequence Detection System (Applied Biosystems) as described previously[26]. Expression of *ABCC11*-mRNA was compared with that of *GAPDH*-mRNA.

Plasma membrane vesicles were prepared from LLC-PK1 cells as described previously[27]. The standard incubation medium contained the plasma membrane vesicles (50 μg protein), 500 μM ^{3}H-labeled cGMP (Amersham), 250 mM sucrose, 10 mM Tris HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM ATP, 10 mM creatine phosphate and 100 μg ml^{-1} creatine kinase in a final volume of 100 μl. Plasma membrane vesicles were incubated at 37 °C for 20 min with ^{3}H-labeled cGMP at different concentrations (0, 5, 10, 20, 50, 100, 250 and 500 μM) in the absence (data not shown) or presence of 1 mM ATP. The amount of ^{3}H-labeled cGMP incorporated into the membrane vesicles was measured by a rapid filtration technique[28]. ATP-dependent cGMP transport was measured by the difference in the radioactivity incorporated into the vesicles in the presence and absence of ATP.

URLs. Haploview program (http://www.broad.mit.edu/mpg/haploview/index.php); International HapMap Project (http://hapmap.jst.go.jp/index.html.en); NCBI GenBank (http://www.ncbi.nih.gov/GenBank); JSNP database (http://snp.ims.u-tokyo.ac.jp); Coriell Institute for Medical Research (http://coriell.umdnj.edu/); dbSNP (http://www.ncbi.nih.gov/SNP/).

Accession codes. GenBank: 134 CA repeat polymorphisms, from AC003034 to AC007728. JSNP: 21 registered SNPs, from IMS-JST085980 to MS-JST141675. dbSNP: newly identified SNPs used in this project, ss49784051 to ss49784077.

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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