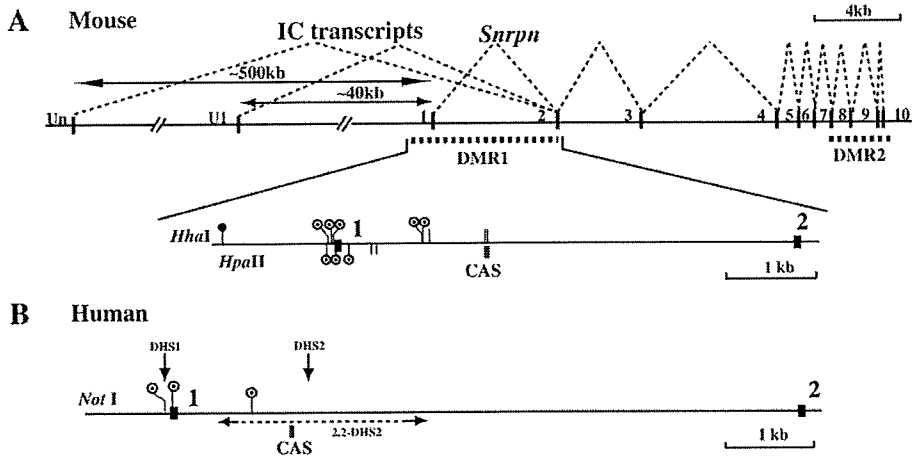

Fig. 1. Genomic organization and the DNA methylation pattern of the mouse (A) and human (B) *Snrpn/SNRPN* locus. Black and gray boxes indicate exons and the CAS region. (A) IC transcripts overlap with *Snrpn* except exon 1. Spliced exons except U exons in IC transcripts are omitted. Short vertical lines above and below the horizontal line indicate 8 *Hha*I and 5 *Hpa*II sites in DMR1, respectively (GenBank accession. no. AC167813). (B) Short vertical lines above the horizontal line indicate 3 *Not*I sites in DMR1. Solid and dotted circles indicate biallelically methylated sites and maternal allele-specific methylated sites (Shemer et al. 1997; Lucifero et al. 2002; Glenn et al. 1996). The *Bss*HII site is described as two *Hha*I sites with dotted circles.

gamete (Shemer et al. 1997). Since DMR1 contains the IC, the methylation status of DMR1 is not only associated with paternal-allele-specific expression of *Snrpn* but may be implicated in imprinting control of other genes in the region.

In the human *SNRPN* locus, parental origin-specific nuclease hypersensitive sites (DHSs) have been identified (Schweizer et al. 1999; Rodriguez-Jato et al. 2005). One of the paternal origin-specific DHSs is DHS1 in the *SNRPN* promoter and the other is DHS2 in *SNRPN* intron 1, roughly 1.5 kb downstream of the transcription initiation site (Fig. 1B). Differential methylation of the CpG island flanking DHS1 is well characterized as a maternal allele-specific DMR in the *SNRPN* promoter. The CpG island associated with DHS2 was reported to show maternal allele-specific methylation in lymphoblastoid cell lines (Rodriguez-Jato et al. 2005), and to be distinct and separate from the CpG rich region in intron 1, 0.9 kb downstream of the transcription initiation site, which was previously described as a DMR in fetal tissues (Fig. 1B) (Glenn et al. 1996). Interestingly, an intronic 2.2-kb fragment (2.2-DHS2) associated with DHS2 was found to enhance the activity of the *SNRPN* promoters (Rodriguez-Jato et al. 2005). The especially highly conserved sequence (CAS) in 2.2-DHS2 among human and rodent was identified to have enhancer activity in human (Fig. 1B). Chromatin immunoprecipitation (ChIP) analysis revealed that the CAS showed paternal chromatin-specific interaction with transcription factors (Rodriguez-Jato et al. 2005). These data suggest that the CAS may play a critical role in activating the paternally expressed imprinted genes in the domain. It remains unknown whether such an allele-specific interaction with transcription factors depends on allele-specific DNA methylation in the CAS and how imprinted transcripts in the *SNRPN* locus can be controlled by the enhancer activity of the CAS.

To investigate further functions of the CAS in the imprinting domain, we analyzed the methylation status of the mouse CAS and a methylation effect on the enhancer activity of the CAS. Our results show that the *Snrpn* promoter region in DMR1 is a germline DMR as previously reported (Shemer et al. 1997), whereas the mouse CAS is a secondary DMR, which is acquired in a tissue-specific manner during development and has the methylation-sensitive activator function.

2. Materials and methods

2.1. Tissues and cells used

All procedures in mice were performed with approval from the Nagasaki University Institutional Animal Care and Use Committee. F1 hybrid mice were obtained by mating C57BL/6 females with PWK males (C57BL/6×PWK), and vice versa (PWK×C57BL/6). Methods of primary cultures of cortical neurons, glial cells and embryonic fibroblasts were described elsewhere (Yamasaki et al. 2005). Oocytes and sperm collections were performed as described elsewhere (Mapendano et al. 2006; Yoshida et al. 1995).

2.2. DNA extraction from gametes, cells and tissues

To prepare oocyte DNA, about 400 pooled oocytes were resuspended in 160 μL of 10 mM Tris–HCl (pH 8.0), 10 mM EDTA, 150 mM NaCl, 0.1% SDS, 2 μg of λ DNA, and 40 μg of proteinase K and incubated for 3 h at 55°C, then extracted with phenol-chloroform and precipitated with ethanol. Blastocyst DNA was prepared from about 10–15 blastocysts with the same procedure. Sperm DNA was isolated by the two-digestion method as described (Yoshida et al. 1995). DNA from other embryos, tissues and human blood was extracted as described elsewhere (Yamasaki et al. 2005).

2.3. Sodium bisulfite treatment and sequencing

Sodium bisulfite treatment was carried out using EZ DNA Methylation-Gold kit (Zymo Research, Orange, CA). PCRs were carried out using bisulfite-treated DNA and each primer set. The following primer pairs were used for amplification: Snrpn-outsideF/Snrpn-outsideR for DMR1 (Lucifero et al. 2002); mCAS-F/mCAS-R for the mouse CAS; hCAS-F/hCAS-R for the human CAS. Primer sequences are as follows: mCAS-F 5′-TGGGGAGGGGTTTATTGTTTTG-3′; mCAS-R 5′-ATAACATCCTAAATTTTAT CAAAATCAT-3′; hCAS-F 5′-TTGGGAAT-TAGGTTTTGGAAGGTT-3′; hCAS-R 5′-ACCTACCCCCTCCCCACTAAC-3′. The amplification protocol was as follows: denaturation at 94 °C for 6 min, followed by 40 cycles at 94 °C for 1 min, 55 °C for 2 min, 72 °C for 2 min, and final elongation at 72 °C for 10 min. PCR products were ligated into PCR2.1 vector by TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) and sequenced on ABI PRISM Model3100.

2.4. Reporter gene construction, methylation, and reporter gene transfection assay

The *Snrpn* promoter regions and the CAS were amplified with following primers: SnrpnL-F/SnrpnL-R for the *Snrpn* promoter; SMP-F/SMP-R for the SMP; U1-F/U1-R for the U1 promoter; mCASL-F/mCASL-R for the CAS. Primer sequences are as follows. SnrpnL-F 5′-

AAGCCCTGTCTCTAAAACCAAC-3′, SnrpnL-R 5′-CTTCCTCGCTCCATTGC-GTTG-3′, SMP-F 5′-CGAGCTCAAATGTGCGCATGTGCAGCC-3′, SMP-R 5′-GCTCGAGCTTCCTCGCTCCATTGCGTTG-3′, U1-F 5′-CGAGCTCATT-CATCACAATGAAAATCAATA-3′, U1-R 5′-CCGCTCGAGCTTGGTTGCTG-CATTGCCTTG-3′, mCASL-F 5′-AGATCTAAGGGGTCGTGTCGCATGTC-3′, mCASL-R 5′-AGATCTGTATACGCCATGCTGCGCC-3′.

PCR products were directly ligated into PCR2.1 vector by TOPO TA Cloning Kit, followed by *Sac*I /*Xho*I double digestion for the *Snrpn* promoter, SMP and the U1 promoter, and by *Bgl*II digestion for the CAS. The *Sac*I/*Xho*I digested products were cloned into the *Sac*I/*Xho*I sites upstream of the firefly luciferase gene and the *Bgl*II digested product was into the *Bam*HI site downstream of the firefly luciferase gene of pGL4.10 (Promega, Madison, WI). *In vitro* methylation of the constructs, plasmids (10 μg) were incubated for 1 h at 37°C in the presence or absence (control, mock) of *Hha*I and *Sss*I methylase (New England BioLabs, Beverly, MA). Plasmids were transfected into Neuro2a cells, using Lipofectamine 2000 reagent (Invitrogen). The pGL4.70, encoding the renilla luciferase gene (Promega) was cotransfected as an internal control for transfection efficiency. Transfected cells were harvested after 24 h and used for measuring firefly and renilla luciferase activities in Dual-Luciferase Assay system (Promega). Reporter activity was normalized by calculating the ratio of firefly to renilla values. For each construct, the average and standard error of the means were calculated in 6 independent transfections.

3. Results and discussion

3.1. The mouse CAS shows developmental stage-specific methylation and maternal allele-specific methylation in the brain

It has been confirmed by many experiments that maternal allele-specific DNA methylation of the *Snrpn* promoter region in DMR1 originates from the egg and is maintained throughout development (Shemer et al. 1997). However, the methylation pattern of the *Snrpn* intron 1 in DMR1 has not been precisely analyzed except in several recognition sites of methylation-sensitive restriction enzymes, *Bss*HII and *Hha*I (Fig. 1A) (Shemer et al. 1997; Gabriel et al. 1998). We first performed methylation analysis of the *Snrpn* promoter region and the mouse CAS in the *Snrpn* intron 1, which is located ~1.8 kb downstream of the *Snrpn* transcription initiation site. Parental origin of the alleles was identified by polymorphic sites in F1 hybrids between C57BL/6 and PWK strains (divergent strains of *Mus musculus*). Allele-specific methylation of 16 CpGs in the promoter and 7 CpGs in the CAS was examined using polymorphisms in PCR products of bisulfite-modified DNA. Sequencing of clones from the PCR products revealed maternal allele-specific methylation of the promoter region in the gametes and somatic tissues (data not shown) as previously reported (Shemer et al. 1997), whereas the CAS showed a different methylation pattern depending on tissues (Fig. 2). In oocytes and sperm, all 7 CpGs in the CAS were not methylated (Fig. 2A). In blastocysts, the maternal allele was slightly methylated, and completely methylated in the whole embryo at embryonic day (E) 9.5. In contrast, the paternal allele was not methylated in blastocysts and the whole embryos at E9.5, and moderately methylated in the embryonic liver and muscle at E15. In the embryonic brain, the paternal allele was almost unmethylated. In the adult brain, the CAS was not methylated on the paternal allele, while in the adult liver, muscle, kidney, and blood, they were biallelically methylated (Fig. 2B). These data indicate that the CAS is a secondary DMR, which is acquired in a tissue-specific manner during development. These results do not conflict with the previous methylation profile of DMR1, reported by Shemer et al. (1997). They detected differential methylation of the *Hha*I site in the CAS in ES cells and brains. In their Southern blot, the CAS is exclusively unmethylated in androgenetic ES cells, while in the parthenogenetic ES cells, the CAS is not completely methylated, although they concluded the maternal allele-specific methylation. The reason why the CAS in parthenogenetic ES cells showed almost methylated might be that the maternal

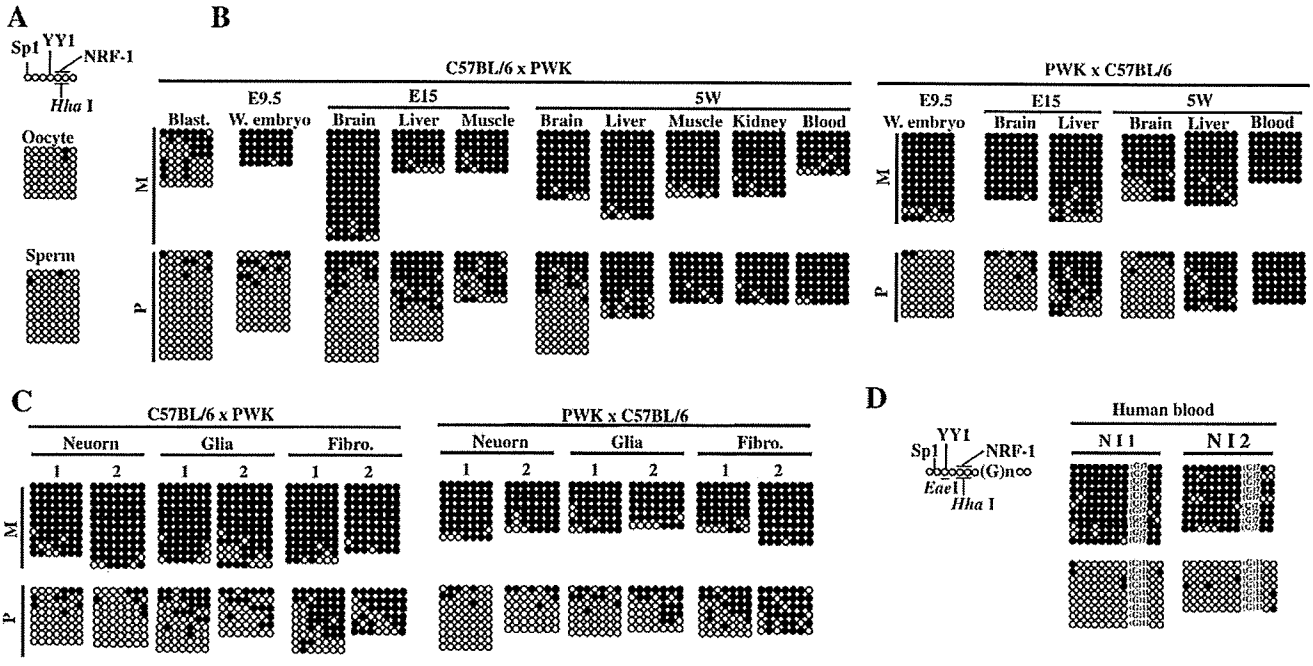

Fig. 2. Methylation of CpG dinucleotides in the CAS in gametes, tissues and culture cells in mice (A, B, C) and in human lymphocytes (D). Each row of dots represents the series of CpGs in an individual sequence molecule, in which methylated CpGs are shown as solid circles and unmethylated CpG as open circles. (A) Mouse oocytes and sperm. The properties of the mouse CAS are displayed at the top of the figure. (B) Embryonic and adult tissues of F1 hybrid mice. Blast.: Blastocyst, W.embryo: Whole embryo, M: maternal allele, P: paternal allele. (C) Culture cells. Two independent sets (1, 2) of each type of culture cells were used for methylation analysis in the CAS. Fibro. : embryonic fibroblasts. (D) Human peripheral bood from normal individuals (NI1 and NI2). The properties of the human CAS are displayed at the left of the figure.

allele in the CAS tends to rapidly acquire DNA methylation after the genome-wide demethylation, even if the parthenogenetic ES cells keep their multipotency during *in vitro* culture.

3.2. Allele-specific methylation of the mouse CAS is not neuron-specific in culture cells

In the embryonic and adult brains, most clones from the paternal alleles at the mouse CAS were not methylated but small numbers of clones were methylated, especially in the F1 hybrid (C57BL/6×PWK) brain. To know the cell origin of the methylated clones, we analyzed the methylation status in cultured neurons, glial cells and embryonic fibroblasts, which were separately cultured from embryonic tissues at E15.5 (Yamasaki et al. 2005). Prior to the analysis, we confirmed by immunostaining with the brain precursor, neuronal and glial makers that over 95% of the two cultured cell types were postmitotic neurons and astrocytes, respectively (data not shown). In neurons and glial cells, most clones from the paternal allele were not methylated, but more methylated clones were detected in glial cells than in neurons, while in the embryonic fibroblasts, many but not all of the paternal clones were methylated (Fig. 2C). We could not detect distinct differences in the methylation profile between cultured neurons and glial cells, as we previously reported in *Igf2r* DMR2 (Yamasaki et al. 2005). Although we could not completely deny the possibility that the tissue-specific methylation profile might not be stably established during *in vitro* differentiation in the embryonic cell culture, these data suggest that the paternal allele escapes methylation during neurogenesis and gliogenesis.

3.3. The human CAS shows allele-specific methylation in the normal lymphocyte

The methylation pattern of the human CAS has previously been reported using genomic DNA from the cell lines derived from uniparental disomy (UPD) patients of PWS and AS (Rodriguez-Jato et al. 2005). Because our bisulfite methylation analysis revealed that the CAS is biallelically methylated in most adult tissues including the lymphocytes in mice, we tried to confirm the methylation status of the human CAS in the normal lymphocyte. A G-nucleotide number polymorphism (Gn; n=7,11) was used to differentiate parental alleles in two normal individuals. We could not identify the parental origin of the alleles because no parental DNA was available, however, the human CAS showed allele-specific methylation in the human peripheral blood lymphocyte (Fig. 2D). We also examined allele-specific methylation in tissues of seven fetuses, but failed to prove it because we could not find any polymorphic sites flanking the CAS in the samples. Although based on a limited number of samples, our data about allele-specific methylation in the normal lymphocyte, in addition to the previous report about the maternal allele-specific methylation in the cell line of UPD patients, strongly suggests that the human CAS is a DMR in differentiated tissues.

3.4. The mouse CAS has a methylation-sensitive activator function

The human CAS was reported not only to activate the *SNRPN* promoter, but also the heterologous promoters in transient expression assays (Rodriguez-Jato et al. 2005). To see if the mouse CAS also has such a promoter activating function, transient expression assays were performed in mouse neuroblastoma Neuro2a cells (Fig. 3). The entire 80 bp of the mouse CAS was inserted to luciferase reporter constructs that include a 754-bp segment of the *Snrpn* promoter (from positions −679 to +75) or a 159-bp segment of the *Snrpn* minimal promoter (SMP) (from positions −84 to +75) (Hershko et al. 1999). Constructs in which the mouse CAS was cloned downstream from the mouse *Snrpn* promoter or the SMP showed approximately 3.5-fold increase in reporter activity, compared with control constructs lacking the mouse CAS. Similar results were obtained independent of orientation and position of the CAS in Neuro2a (data not shown).

Since the mouse CAS shows the tissue-specific and developmental stage-specific methylation, an effect of methylation on the promoter activation function was examined. The methylation effect on two *Hha*I sites in the mouse CAS was analyzed in the constructs with the *Snrpn* upstream promoter U1 (Fig. 1A), because the U1 promoter sequence has no *Hha*I sites. The U1 promoter region was cloned into a luciferase reporter construct that included the CAS positioned downstream of the reporter in the forward orientation. Transient expression assays revealed that the U1 promoter was activated by the CAS relative to a construct lacking the CAS (5.5 fold). *In vitro* methylation by *Hha*I methylase prior to transfection of Neuro2a cells activated the U1 promoter activity to a less extent (2 fold) than no methylation in the CAS. *In vitro* methylation of the five CpGs within the U1 promoter and seven CpGs within the CAS by *Sss*I methylase resulted in a complete

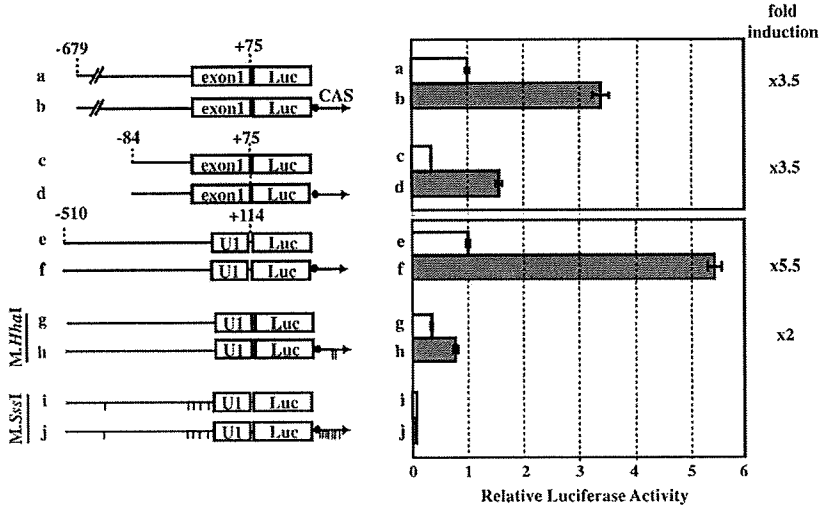

Fig. 3. Methylation-sensitive enhancer activity of the mouse CAS. Reporter constructs with the *Snrpn* promoter (a, b), the *Snrpn* minimal promoter (c, d), and the U1 promoter (e, f, g, h, i, j) lacking or carrying the mouse CAS (arrow) were assayed by transient expression assays. In the constructs (g, h, i, j), *in vitro* methylation with *Hha*I (g, h) or *Sss*I (i, j) methylases was performed. Short vertical lines below the lines and arrows (h, i, j) indicate methylated CpGs. The constructs containing the *Snrpn* promoters (a), or the U1 promoter (e) without the CAS are arbitrarily assigned the value 1. The fold increase induced by the CAS or *in vitro* methylated CAS is indicated on the right.

shut-off of the promoter activity. These data indicate that the mouse CAS has the promoter activation function and its activity depends partially on the methylation status of the *Hha*I sites in the CAS.

In the human and rodent CAS, potential binding sites for transcription factors Sp1, YY1, and NRF-1 are highly conserved (Rodriguez-Jato et al. 2005). It is reasonable in our study that methylation of the two *Hha*I sites at the NRF-1 binding sequence directly decreased the enhancer activity because of methylation-sensitive binding of NRF-1 (Smith et al. 2004), however the fact that the CAS with methylated NRF-1 binding site still have enhancer activity suggests other factors including Sp1 and YY1 may coordinately constitute the enhancer complex at the paternal CAS.

In addition to the methylation-sensitive enhancer activity, the evidence that the mouse CAS is not methylated in oocytes and not methylated on the paternal allele in the brain, correlates with tissue-specific expression of IC transcripts, which initiate in U exons (Un) that are distributed in a 500-kb region upstream of *Snrpn* and overlap with *Snrpn* exons except exon 1 (Fig. 1A). In mice, IC transcripts are exclusively expressed in the ovary and brain, especially in oocytes and neurons (Mapendano et al. 2006), where the CAS is unmethylated and differentially methylated, respectively (Fig. 2A, C). The function of IC transcripts remains unknown, but the tissue-specific methylation of the mouse CAS and its methylation-sensitive enhancer activity may control tissue-specific expression of IC transcripts, resulting in the establishment of imprinting in oocytes (Mapendano et al. 2006) and neuron-specific imprinting of *Ube3a* in the brain, possibly by the antisense *Ube3a* transcript as a part of IC transcripts.

On the other hand, in human, there is no simple correlation of expression level of the IC transcripts and the CAS methylation. IC transcripts are expressed in some tissues including adult heart, brain, and ovary, but not in blood (Dittrich et al.1996), where the CAS is differentially methylated (Fig. 2D) and associated with allele-specific histone modifications and interactions with multiple regulatory proteins (Rodriguez-Jato et al. 2005). Such difference in DNA methylation in the CAS and expression of IC transcripts between human and mouse may support divergency of imprinting mechanism among species (Johnstone et al. 2006). The targeted replacement of the mouse PWS-IC with the equivalent human region in mice failed to maintain methylation on the maternal allele in somatic tissues and to protect the upstream genes (*Ndn* and *Mkrn3*) during *de novo* methylation in early embryogenesis (Johnstone et al. 2006). They suggested that the factors responsible for postzygotic maintenance of the imprint have diverged between human and mouse. Because their targeted PWS-IC region contains the CAS, where methylation is postzygotically acquired in mice, methylation status of the substituted CAS might be altered in oocyte and/or in early embryogenesis, resulting in failure of maintenance of imprinting. It is still controversial in human whether the methylation imprint at the *SNRPN* promoter region is established in ovulated oocyte or during/following fertilization (El-Maarri et al. 2001; Geuns et al. 2003), however the timing of DNA methylation acquisition at the substituted CAS in the targeted mouse will clarify the basic difference in imprinting machinery among human and rodents.

In conclusion, we have demonstrated that the *Snrpn* intron 1, which was previously thought to be a part of a germline DMR in mouse, shows developmentally dynamic changes of DNA methylation in mouse, and has the methylation-sensitive enhancer activity. The tissue-specific methylation of the mouse CAS and its methylation-sensitive enhancer activity may control tissue-specific expression of IC transcripts, resulting in the establishment and/or maintenance of imprinting in the *Snrpn* locus.

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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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SHORT COMMUNICATION

A type of familial cleft of the soft palate maps to 2p24.2–p24.1 or 2p21–p12

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Cleft of the soft palate (CSP) and the hard palate are subtypes of cleft palate. Patients with either condition often have difficulty with speech and swallowing. Nonsyndromic, cleft palate isolated has been reported to be associated with several genes, but to our knowledge, there have been no detailed genetic investigations of CSP. We performed a genome-wide linkage analysis using a single-nucleotide polymorphism-based microarray platform and successively using microsatellite markers in a family in which six members, across three successive generations, had CSP. A maximum LOD score of 2.408 was obtained at 2p24.2–24.1 and 2p21–p12, assuming autosomal dominant inheritance. Our results suggest that either of these regions is responsible for this type of CSP.
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Keywords: cleft of the soft palate; genome-wide linkage analysis; submucous cleft palate

INTRODUCTION

Orofacial cleft, one of the most common congenital malformations, is a heterogeneous group of complex traits. Orofacial cleft is classified into two main categories, cleft lip with or without cleft palate and cleft palate isolated (CPI). Both clefting phenotypes can appear to be related to some syndromes (syndromic orofacial cleft) or not be related to syndromes (nonsyndromic orofacial cleft). CPI is considered genetically distinct from cleft lip with or without cleft palate, on the basis of epidemiological evidence and the different developmental timing of lip and palate formation. Recent molecular genetic studies[1–5] have identified genes or loci that are responsible for CPI. However, fewer genes and/or loci-associated CPI have been reported in comparison with cleft lip with or without cleft palate.[6]

CPI is mostly classified into two subtypes morphologically: cleft of the hard palate (CHP) and cleft of the soft palate (CSP).[7] Submucous cleft palate (SMCP) is a small subgroup in the CPI. SMCP manifests with bifid uvula, separation of the muscle with an intact mucosa and a bony defect in the posterior edge of the hard palate.[8] Both CHP and CSP are caused by a failure of fusion of the palatal shelves, but little is known about the cause of the difference in their phenotypes. Christensen *et al.*[9] suggested that CHP and CSP might be etiologically distinct. Although patients with CSP have serious problems in speech and deglutition, as well as CHP, there have been no detailed genetic studies performed.

We recently encountered a Japanese family that included five CSP patients and one SMCP patient. The aim of this study was to identify the CSP/SMCP predisposing locus in this family using genome-wide single-nucleotide polymorphism (SNP)-based linkage analysis.

MATERIALS AND METHODS

Family and patients

A Japanese family included five patients (I-2, II-2, II-3, III-1 and III-2) with CSP and one patient (II-5) with SMCP across three generations (Figure 1). Two patients (II-2 and II-3) were monozygotic twins. The phenotypes of two patients (III-1 and II-5) were shown in Figure 2. All patients had no other symptoms such as mental retardation, and all family members were examined by one or two well-trained dentists.

The disease in the family was consistent with an autosomal dominant mode of inheritance. Blood samples were obtained with written informed consent from 15 cooperative family members (Figure 1). The study protocol was approved by the committee for ethical issues on the Human Genome and Gene Analysis of Nagasaki University.

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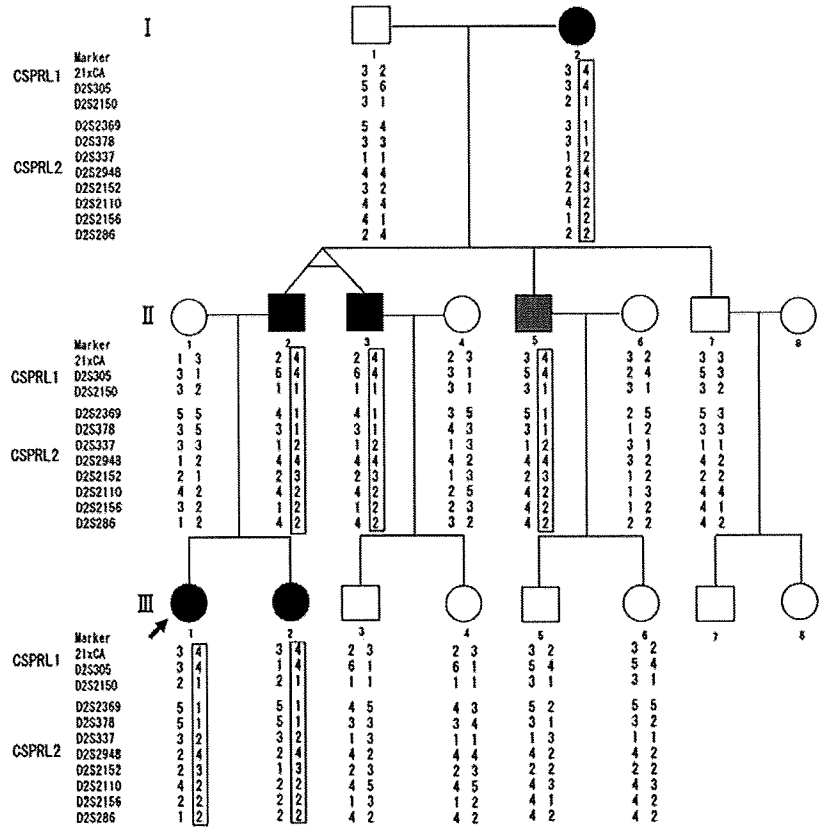

Figure 1 Family tree with haplotypes at 2p24.2–24.1 (CSPR1) and 2p21–p12 (CSPR2). Black closed, gray closed and open symbols indicate affected with cleft of the soft palate (CSP), affected with subcutaneous cleft palate (SMCP) and unaffected, respectively. An arrow indicates the proband. Genotypes of microsatellite markers defining the candidate intervals are shown below each individual. Boxed haplotype indicates possibly disease-associated haplotype.

SNP genotyping and linkage analysis

Genomic DNA was extracted from peripheral blood lymphocytes of the 15 members, using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). Their genotypes were determined using a GeneChip Human Mapping 10K 2.0 Xba Array (Affymetrix, Santa Clara, CA, USA). We used MERLIN software[10] to analyze compiled pedigree data sets. Mendelian errors were detected by PEDCHECK,[11] and SNPs with Mendelian error were not used in the data analysis. LOD scores were calculated under a parametric autosomal dominant model in which penetrance was set to 1.0 and disease allele frequency was 0.00001. As CSP and SMCP can be categorized together because of their similar anatomical features,[9] the patient with SMCP and the patients with CSP (II-5) were classified as 'affected' for linkage score calculations.

To confirm the result of linkage data using the GeneChip Human Mapping 10K 2.0 Xba Array, we performed a two-point linkage analysis using microsatellite markers by the method described elsewhere.[12] The two-point LOD score was calculated using MLINK program.[13]

RESULTS AND DISCUSSION

In the assay with the 10K-Array, the GeneChip call rates varied from 92.18 to 99.42% (with a mean of 97.54%). Two regions, 2p24.2–p24.1 (CSP region 1: CSPR1), a 4.5-Mb interval between rs1545497 and rs1872325, and 2p21–p12 (CSP region 2: CSPR2), a 34.5-Mb segment between rs940053 and rs310777, were CSP candidate loci with a maximum LOD score of 2.408 (Figure 3). The LOD scores of all other regions were below 1.000. Two-point LOD scores using microsatellite markers showed the same scores (2.408); therefore, the result of linkage analysis from SNP genotyping was reconfirmed (haplotype using microsatellite markers was shown in Figure 1). It is thus likely that a gene having a role in palatal fusion is located within either CSPR1 or CSPR2.

On the basis of our knowledge of oral palate development, we chose nine genes from the candidate CSP regions and performed mutation analysis. Of the nine candidate genes, three were from CSPR1: growth/differentiation factor 7 (*GDF7*), matrilin 3 (*MATN3*) and member B of the Ras homolog gene family (*RHOB*). The other six genes were from CSPR2: calmodulin 2 (*CALM2*), bone morphologic protein 10 (*BMP10*), sprouty-related EVH1 domain-containing protein 2 (*SPRED2*), transforming growth factor, alfa (*TGFA*), ventral anterior homeobox 2 (*VAX2*; 2p13.3) and stoned B-like factor/stonin 1 (*STON1*). Most of these genes are concerned with bone development, and with the transforming growth factor and mitogen-activated protein kinase signaling pathways, or are transcription factors related to homeobox genes. However, no pathogenic mutation was found within any of its exons or intron–exon boundaries of all nine genes.

To detect structural genomic alterations that may cause CSP within the candidate regions, we performed copy number analysis with the proband's DNA using the Genome-Wide Human SNP Array 5.0 (Affymetrix). Although several copy number alterations were detected (data were not shown), all were already registered as copy number variations on the UCSC Genome Browser (http://genome.ucsc.edu/) and none of them coincided with regions with positive LOD scores.

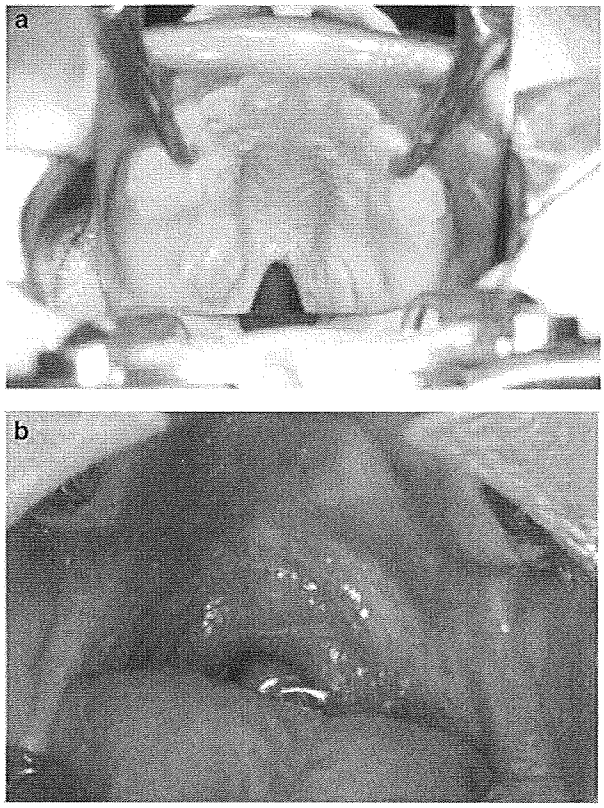

Figure 2 Views of palates. The palate of individual III-1 with CSP (**a**) showing a cleft limited to the soft palate, and that of individual II-5 with SMCP (**b**) showing a translucent zone in the soft palate resulting from a separation of the muscle.

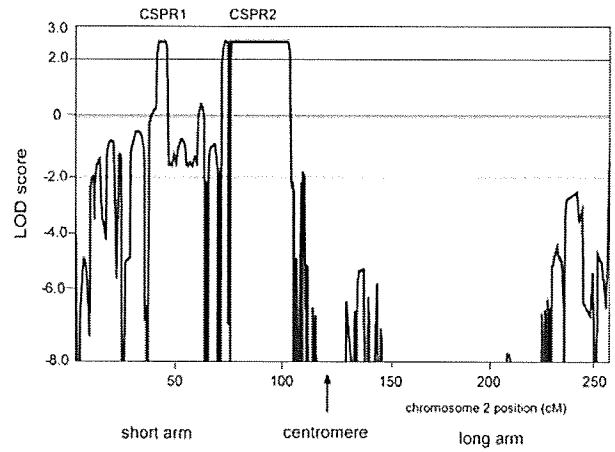

Figure 3 Multipoint LOD scores on chromosome 2. A 4.5-Mb (physical position, 18281893–22775527) interval from rs1545497 to rs1872325 corresponds to CSPR1, and a 34.5-Mb interval (45834656–80355227) from rs940053 to rs310777 corresponds to CSPR2.

In conclusion, this is the first report of a whole-genome linkage analysis scan for CSP. Although the LOD scores calculated are not high enough to assign the disease locus definitively, our data suggest that it lies at either 2p24.2–24.1 or 2p21–p12.

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RESEARCH LETTER

AMERICAN JOURNAL OF medical genetics PART A

A *ZRS* Duplication Causes Syndactyly Type IV With Tibial Hypoplasia

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TO THE EDITOR:

Point mutations in the highly conserved sequence of the long-range sonic hedgehog (*SHH*) regulator (*ZRS*) at 7q36.3 cause preaxial polydactyly (PPD) [Lettice et al., 2002, 2003; Gurnett et al., 2007; Furniss et al., 2008]. Genomic duplications of *ZRS* cause the triphalangeal thumb-polysyndactyly syndrome (TPTPS) and syndactyly type IV (SD4) [Klopocki et al., 2008; Sun et al., 2008]. SD4 (OMIM 186200) is a rare disorder first described by Haas [1940] [see also Gillessen-Kaesbach and Majewski, 1991; Rambaud-Cousson et al., 1991; Sato et al., 2007; Sun et al., 2008]. Patients with this condition have complete syndactyly of all fingers with polydactyly, and cup-shaped hands due to flexion of fingers. We previously assigned SD4 to a 17.39-cM region at 7q36 in a Chinese SD4 family with tibial hypoplasia using linkage and haplotype analyses, however, direct sequencing of the patients' DNA showed no pathogenic mutation in *LMBR1*, *SHH* or the conserved sequence of *ZRS* [Sato et al., 2007]. Here, we confirm that SD4 with tibial hypoplasia is caused by a genomic duplication involving *ZRS*, which may lead to abnormal regulation of *SHH* expression.

The family studied here is the same one reported previously [Sato et al., 2007], including eight affected individuals who had complete syndactyly of all fingers, polydactyly, cup-shaped hands, and occasionally hypoplastic tibiae, which lead to a diagnosis of Haas type mirror-image polydactyly of hands and feet (SD4) with tibial hypoplasia. DNA samples were extracted from six affected and five unaffected individuals of the family after obtaining written informed consent in accordance with study protocols approved by the University Ethics Committees.

To test whether SD4 with tibial hypoplasia is associated with a copy number mutation, we used a qPCR assay to determine RCN of *ZRS* based on the ΔΔCt method [Sun et al., 2008] and detected a *ZRS* duplication in one affected individual (III-5) of the SD4 family. Using this qPCR assay, we confirmed that the duplication co-segregated with the limb phenotype in all affected relatives but was not detected in any unaffected relatives or in 50 unrelated control Han Chinese. To determine the size of the genomic duplication, we

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performed qPCR assays as described previously [Sun et al., 2008] in two affected persons, III-5 (with tibial hypoplasia) and IV-3, using a cut-off RCN of 1.3; we identified a duplication of at least 105 kb spanning from nt. 156232366 to nt. 156337864 which contains *ZRS* (Fig. 1A).

Our multiple qPCR assays detected the minimal duplication but could not give precisely the breakpoints in the case. Therefore, we performed copy number and LOH analyses on three samples of IV-1 (with tibial hypoplasia), IV-3 and IV-2 (unaffected) using Illumina HumanHap550-Duo (http://www.Illumina.com). A duplication covering a 97-kb segment from nt. 156240230 to nt. 156336835 and involving *LMBR1* (Fig. 1B) was found in both patients, while no copy number variant in this region was detected in IV-3. Two SNPs flanking both sides of the duplication, rs6956930 (nt. 156230391) and rs2365750 (nt. 156345168), are not duplicated, indicating the centromeric breakpoint region of 1.975 kb from nt. 156230391 to nt. 156232366, and the telomeric breakpoint region of 7.3 kb from nt. 156337864 to nt. 156345168.

Based on the observations of an SD4 family, Rambaud-Cousson et al. [1991] suggested that SD4 is in fact a complex entity which can include a variety of lower limb malformations in addition to its typical abnormalities. We also suggested previously that SD4 with tibial hypoplasia might be a severe clinical subtype of SD4 [Sato et al., 2007], which has been genetically confirmed in the present study. Moreover, three abnormalities, that is, triphalangeal thumb, mirror polydactyly and tibial hemimelia tend to overlap among SD4, PPD, TPTPS, THPTTS (tibial hemimelia polysyndactyly triphalangeal-thumb syndrome) and mirror polydactyly with tibial hemimelia, all of which have been mapped to 7q36 [Heutink et al., 1994; Tsukurov et al., 1994; Zguricas et al., 1999]. Therefore, Kantaputra and Chalidapong [2000] proposed that THPTTS, TPTPS, PPD-2/3, and Haas-type syndactyly (SD4) are pathogenetically related. Our work, together with these previous reports [Lettice et al., 2003; Klopocki et al., 2008; Sun et al., 2008], confirmed that TPTPS and SD4 are allelic with PPD2/PPD3 (Table I) and indicated that THPTTS, TPTPS, PPD-2/3, and SD4 represent a phenotypic spectrum caused by various mutations of *SHH* or its regulator, *ZRS*.

Recently, various genomic duplications involving *ZRS* have been reported in seven families with TPTPS and/or SD4 in at least two distinct ethnic groups [Klopocki et al., 2008; Sun et al., 2008]. Sun et al. [2008] suggested that the smallest region of overlap (SRO) among various sizes of duplications in families they collected should be the critical region for PPD and SD4, which was strongly supported by our SD4 family. In comparison with seven other reported families with a duplication involving *ZRS* [Klopocki et al., 2008; Sun et al., 2008], the present family, even though with the smallest duplication, shows the most severe lower limb malforma-

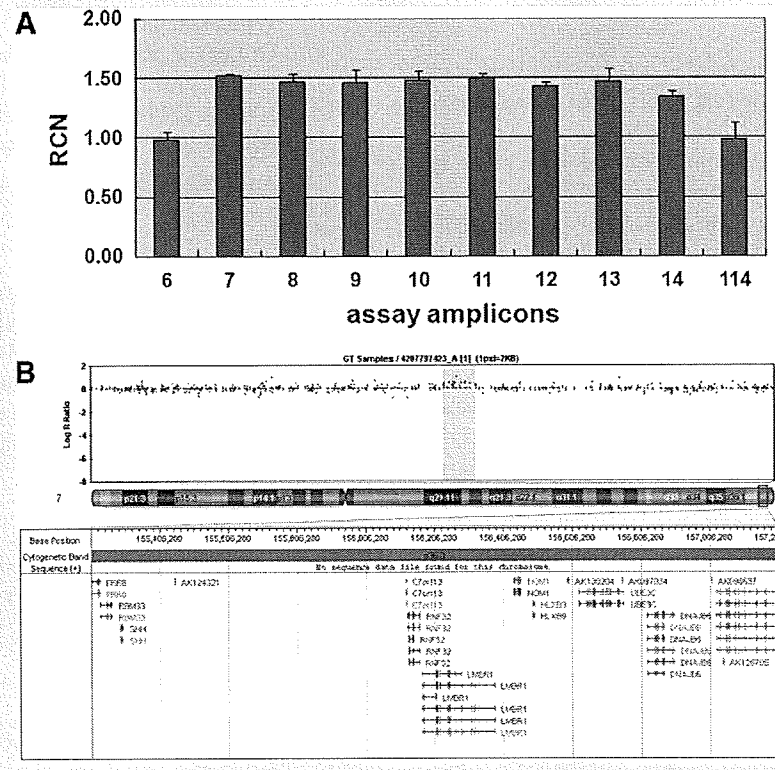

FIG. 1. Detection of *ZRS* duplication. A: The qPCR assay confirming a *ZRS* duplication in an affected individual (III-5). The duplication was seen as a 1.5-fold normalized RCN. B: Plots of the copy number for individual SNP loci along chromosome 7q by Illumina HumanHap550 Genotyping Chips, showing a 96,605 bp duplication from nt 156,240,230 to nt 156,336,835 at 7q36.3 in one affected member with tibial hypoplasia (IV-1). Schematic diagram of the known gene, *LMBR1*, affected by this duplication is depicted below the plots.

TABLE I. Clinical Phenotypes of 16 Reported Families With Mutations of *ZRS*

Mutations types	7 Point mutations	8 Duplications		
Clinical diagnosis, families [Refs.]	PPD2/3, 8 [1–3]	SD4, 1 [4]	SD4-severe, 1 [5]	TPTPS, 6 [4, 6]
Polydactyly				
Pre-axial	+	+	+	+
Post-axial	−	+	+	+
Hand	+	+	+	+
Foot	+	−	+	+
Mirror image	−	+	+	−
Triphalangeal thumb				
Single	+	−	−	+
Duplication	+	+	+	+
Syndactyly				
Complete	−	+	+	+
Partial	−	+	+	+
Cutaneous	−	+	+	+
Osseous	−	−	−	+
Hand	−	+	+	+
Foot	−	−	+	+
Cup-shaped hand	−	+	+	−
Tibial hypoplasia	−	−	+	−

1. Lettice et al. [2003]; 2. Gurnett et al. [2007]; 3. Furniss et al. [2008]; 4. Sun et al. [2008]; 5. Present study; 6. Klopocki et al. [2008].

tions. This may indicate that there is no correlation between phenotypic severity and the extent of duplications and in turn implies that a critical region for the disorders may exist within the smallest duplication. For detection of the copy number mutation, while the multiple qPCR assay used in the present study is rapid, sensitive and cheap, the SNP array method provides more precise results helpful for breakpoint mapping.

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ORIGINAL ARTICLE

Japanese map of the earwax gene frequency: a nationwide collaborative study by Super Science High School Consortium

The Super Science High School Consortium[1]

Wet/dry types of earwax are determined by the c.538G>A single-nucleotide polymorphism (SNP) in the *ABCC11* gene; GA and GG genotypes give the wet type and AA the dry type. The Japanese population may have a dual structure comprising descendants of mixtures between the ancient 'Jomon' and 'Yayoi' populations. We hypothesized that the dry type was introduced by the Yayoi people to the Jomon population where the wet type was predominant, and as the mixture of the two populations has not yet been complete, the allele-A frequency (f^A) would even now be higher along a putative Yayoi man's peopling route within Japanese islands. To know the frequency, a nationwide Super Science High School (SSH) Consortium collected 1963 fingernail samples of pupils/students from at least one high school/university in every prefecture. All further procedures, DNA extraction, SNP genotyping and gene frequency estimation, were carried out by trained SSH pupils. Although the allele-A frequency varied among the 47 prefectures, the Gifu/Kyoto and Okinawa prefectures showed the highest and lowest values, respectively. Areas with high frequencies included Northeastern Kyushu, Northern Shikoku and Kinki districts, showing a belt-like zone, whereas those with low frequencies other than Okinawa were the Southwestern Kyushu, Hiroshima prefecture and Tohoku districts. The f^A value in Kinki district was statistically higher than those in prefectures westward and east-northward from it. The result may provide another line of evidence supporting a possible route of the Yayoi-man's peopling in Japan.
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Keywords: *ABCC11* gene; earwax type; gene frequency; Japanese history; Japanese population

INTRODUCTION

Human earwax is classified into two distinct phenotypes: wet and dry types. The wet earwax is a real secretory product from the ceruminous apocrine gland, whereas the dry type is the phenotype of lacking or reduced ceruminous secretion.[1,2] In 1907, Kishi[3] first described the different nature of earwax between the Japanese and Europeans. Adachi[4] studied earwax and axillary odor phenotypes in the Japanese and nearby ethnic populations, and not only found that the dry type is more frequently seen than the wet type among the Japanese but also that the wet type is linked to axillary odor. Matsunaga[5] provided evidence that the human earwax is a bimorphic Mendelian trait, and that the wet type is completely dominant to the dry type. Subsequent studies of various ethnic populations revealed that the dry type is highly specific to East Asians with a frequency of 80–95%, whereas it is rare (0–3%) in populations of European or African origin, and intermediate in values (30–50%) in Southeast Asian, Oceanian, Central Asian and Middle-East populations as well as in native Americans and Inuit people.[6–12]

By a genetic linkage analysis of eight Japanese families, Tomita *et al.*[13] successfully mapped the human earwax gene locus to 16p11.2-q12.1. Yoshiura *et al.*[2] at the same laboratory then identified the earwax determining gene, *ABCC11* (for ATP-binding cassette, sub-family C, member 11). A functional single-nucleotide polymorphism (SNP), c.538G>A (rs17822931), of the gene determines the earwax types; that is, AA homozygotes for allele A at rs17822931 have dry earwax, and GA heterozygotes and GG homozygotes have wet earwax. The allele-A frequencies calculated for 33 different ethnic populations around the world varied but showed a downward cline from the highest areas (the frequency of 1.00) in Shanxi Province (Northern Han Chinese) and Taegu City (Koreans) toward Japan and Southern Asia and toward Central Asia and Europe.[2] This map corresponded to the phenotypical data of earlier studies mentioned above.[4–12] The map may also indicate a possible route of migration and peopling of ancient Northeast Mongoloid with dry earwax.

There are two main models proposed for the origin of the variation in the current Japanese population: the dual-structure model versus the single-origin hypothesis.[14,15] According to the former model, the modern Japanese are descendants of mixtures between at least two populations, the 'Jomon' and 'Yayoi' people (both are named after the ware types they used). The Jomon people formed the native Japanese

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population inhabited widely in Japan islands since 16 000 years ago, and the Yayoi people were those who came over 3000–1800 years ago. Other immigrants, who came over largely from the Korean Peninsula, further joined in the society in the third to eighth centuries. On the other hand, the single-origin hypothesis proposes that the divergence in various districts may have led to the current genetic variation in Japan. On the basis of data from earlier studies,[2,5,9] we favored the dual structure theory and hypothesized that the dry earwax of the Japanese was introduced by the Yayoi people to the wet-type predominant Jomon population background. As the mixture of these populations has not yet been complete, the dry-type allele would be more frequently observed even now along a migration route of the newcomer population within Japan's islands.

As there has been no gene-based Japanese map of earwax types, a nationwide consortium of Super Science High School (SSH) was recently constructed to study the earwax gene frequency in every prefecture of Japan. Here, we report the result of a large-scale collaborative study by the SSH Consortium.

MATERIALS AND METHODS

The consortium composed of 48 SSHs that are located in various prefectures of Japan was established in 2006. The SSHs are natural science oriented high schools that are selected, approved and supported for 5 years by the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan and the Japan Science and Technology Agency (JST). At its first assembly in 2006 in Nagasaki City, the SSH Consortium started a 2-year collaborative study with a common protocol that had been approved by the Committee for the Ethical Issues on Human Genome and Gene Analysis in Nagasaki University and also in the Health Sciences University of Hokkaido.

The consortium collected several (up to 10) fingernail clippings from each of 19–113 pupils and/or teachers from at least one SSH in every prefecture after obtaining written informed consent from them. In some prefectures where an SSH was not available or found it difficult to cooperate for the study, nail samples were obtained from the pupils of other high schools or from adult volunteers in respective prefectures under assist by some universities. The fingernail clippings obtained were put into a plastic tube, and the sample tubes were then numbered anonymously at each school/university and sent by mail to Nagasaki Nishi High School, one of the SSH Consortium members. Pupils of this school and some other SSHs were provided with minimum essential knowledge and methods of molecular genetic analysis of the human earwax gene during their summer/winter vacations by the staff members of the Department of Human Genetics, Nagasaki University Graduate School of Biomedical Sciences.

A total of 1963 nail samples were collected from 55 high schools (48 SSHs and seven other high schools) and five universities (Table 1). The nail clippings were once frozen in liquid nitrogen and crushed into fine pieces manually with scissors, or into fine powder using Multi-beads Shocker (Yasui Kikai, Osaka, Japan). The piece/powder was dissolved in a urea-lysis solution (2 M urea; 0.5% SDS; 10 mM Tris-HCl, pH 7.5; 50 mM EDTA) containing 1 mg ml^{-1} proteinase K and 40 mM DTT at 55 °C overnight. Nail DNA in lysis solution was extracted with the QIAamp DNA Mini Kit (Qiagen, Tokyo, Japan). To genotype at the rs17822931 locus in a large number of samples, PCR was performed by the use of hydrolyzing TaqMan probes and a set of amplification primers. Their sequences (5′–3′) were as follows: CAGTGTACTCGGGCCAG and CAGT GTACTCAGGCCAG for hydrolyzing VIC-labeled TaqMan MGB wet probe and FAM-labeled dry probe, respectively; and CTTCTGGGCATCTGCTTCTG and CAAACCTCACCAAGTCTGCCA for EW-ampF and EW-ampR primers, respectively. Reactions were carried out using TaqMan Universal PCR Master Mix (AppliedBiosystems, Foster City, CA, USA). Thermal cycling was performed initially at 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s and 62 °C for 1 min on LightCycler480 (Roche Diagnostics, Basel, Switzerland). Genotypes were determined according to the FAM/VIC fluorescence intensity ratio (Figure 1). The allele-A frequency was calculated with the formula of $(2A+B)/2C$, where A is the number of 'AA' homozygotes, B is the number of 'GA' heterozygotes and C is the total number of individuals examined.

Table 1 Allele-A frequency at each prefecture

Prefectures	No. of genotypes at rs17822931 AA	GA	GG	Total	Allele-A frequency ± 95% confidence interval
Hokkaido	47	8	0	55	0.927±0.069
Aomori	80	17	1	98	0.903±0.059
Iwate	21	20	2	43	0.721±0.134
Miyagi	17	6	2	25	0.800±0.157
Akita	23	6	0	29	0.897±0.111
Yamagata	38	14	0	52	0.865±0.093
Fukushima	45	18	1	64	0.844±0.089
Ibaragi	73	19	2	94	0.878±0.066
Tochigi	36	10	1	47	0.872±0.096
Gunma	55	19	0	74	0.872±0.076
Saitama	21	9	0	30	0.850±0.128
Chiba	22	7	0	29	0.879±0.119
Tokyo	21	4	0	25	0.920±0.106
Kanagawa	20	7	0	27	0.870±0.127
Niigata	25	9	0	34	0.868±0.114
Toyama	13	6	0	19	0.842±0.164
Ishikawa	21	7	0	28	0.875±0.123
Fukui	42	6	0	48	0.938±0.068
Yamanashi	27	11	2	40	0.813±0.121
Nagano	21	4	1	26	0.885±0.123
Gifu	22	1	0	23	0.978±0.060
Shizuoka	60	18	2	80	0.863±0.075
Aichi	18	6	0	24	0.875±0.132
Mie	11	2	0	13	0.923±0.145
Shiga	21	4	0	25	0.920±0.106
Kyoto	26	1	0	27	0.981±0.051
Osaka	19	4	0	23	0.913±0.115
Hyogo	24	4	0	28	0.929±0.095
Nara	19	4	0	23	0.913±0.115
Wakayama	99	13	1	113	0.934±0.046
Tottori	37	15	2	54	0.824±0.102
Shimane	15	7	0	22	0.841±0.153
Okayama	17	8	0	25	0.840±0.144
Hiroshima	28	22	1	51	0.765±0.116
Yamaguchi	62	18	2	82	0.866±0.074
Tokushima	31	9	0	40	0.888±0.098
Kagawa	41	7	1	49	0.908±0.081
Ehime	40	5	0	45	0.944±0.067
Kohchi	44	16	1	61	0.852±0.089
Fukuoka	21	3	1	25	0.900±0.118
Saga	18	6	1	25	0.840±0.144
Nagasaki	36	6	1	43	0.907±0.087
Kumamoto	37	11	2	50	0.850±0.099
Oita	22	2	0	24	0.958±0.080
Miyazaki	45	5	1	51	0.931±0.070
Kagoshima	19	6	0	25	0.880±0.127
Okinawa	13	11	1	25	0.740±0.172
Total/average	1,513	421	29	1,963	0.878±0.014

High schools and universities that participated in this study are listed in APPENDIX.

RESULTS AND DISCUSSION

This study confirmed that the Japanese population has two distinct earwax types. The genotype detection rate among 1963 nail samples was 99.6%, and the average allele-A frequency (f^A) among 47 prefectures was 0.878. Thus, the dry-type frequency among the Japanese is 77.1%, which is given as the square value of f^A by assuming

the Hardy–Weinberg equilibrium, being comparable with those estimated from earlier phenotypical studies of earwax.[4,5] The f^A value with 95% confidence interval in each prefecture is shown in Table 1. A Japanese map by prefecture on which these values are depicted in color, that is, the lighter color, the higher frequency, is shown in Figure 2. The map shows the highest and the lowest allele-A frequencies in the Gifu/Kyoto prefectures and in the Okinawa prefecture, respectively. Areas with relatively high frequencies included Northeastern Kyushu, Northern Shikoku along the Seto Inland Sea, and Kinki district between the Hyogo and Gifu prefectures, showing a belt-like lighter-color zone, whereas those with relatively low frequencies other than the Okinawa prefecture were the Southwestern Kyushu, Chugoku (around Hiroshima prefecture) and Tohoku districts. The f^A value in Kinki district (Hyogo, Osaka, Nara, Wakayama, Kyoto and Shiga prefectures) is statistically higher than those in prefectures westward from Kinki (P=0.0030 when including Tokyo and Hokkaido, and P=0.0016 when excluding them, using Mann–Whitney's U-test) and east-northward (P=0.0053 when including Okinawa, and P=0.067 when excluding it). The f^A values look higher in the westward prefectures than that in the east-northward prefectures, but they were not significantly different (P=0.7308 and P=0.8360, when including and excluding Tokyo/Hokkaido and Okinawa prefectures, respectively, using Mann–Whitney's U-test).

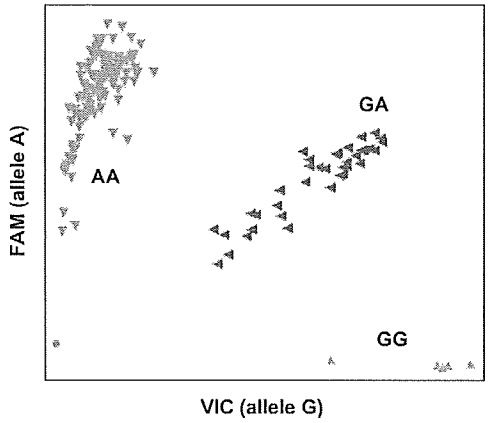

Figure 1 Three different genotypes at the rs17822931 locus by the TaqMan PCR method. 'AA', 'GA' and 'GG' are AA homozygotes (phenotypically dry type) GA heterozygotes (wet type) and GG homozygotes (wet type), respectively.

As far as the earwax variation among the Japanese is concerned, the results in this study cannot absolutely rule out either the dual structure model or the single-origin hypothesis. Our earlier analysis on three SNP sites (rs17822931–rs6500380–ss49784070) around the *ABCC11* locus showed a common haplotype in each of dry-type individuals and wet-type individuals.[2] Therefore, considering these earlier haplotyping data suggesting a founder effect of the allele-A,[2] the present data may favor the dual-structure model.

According to our initial hypothesis of this study, prefectures with lighter color would indicate the areas with more descendants of the Yayoi population, whereas those with darker color indicate the areas with more descendants of the Jomon population. The earwax gene frequency map tended to correspond to the distribution for

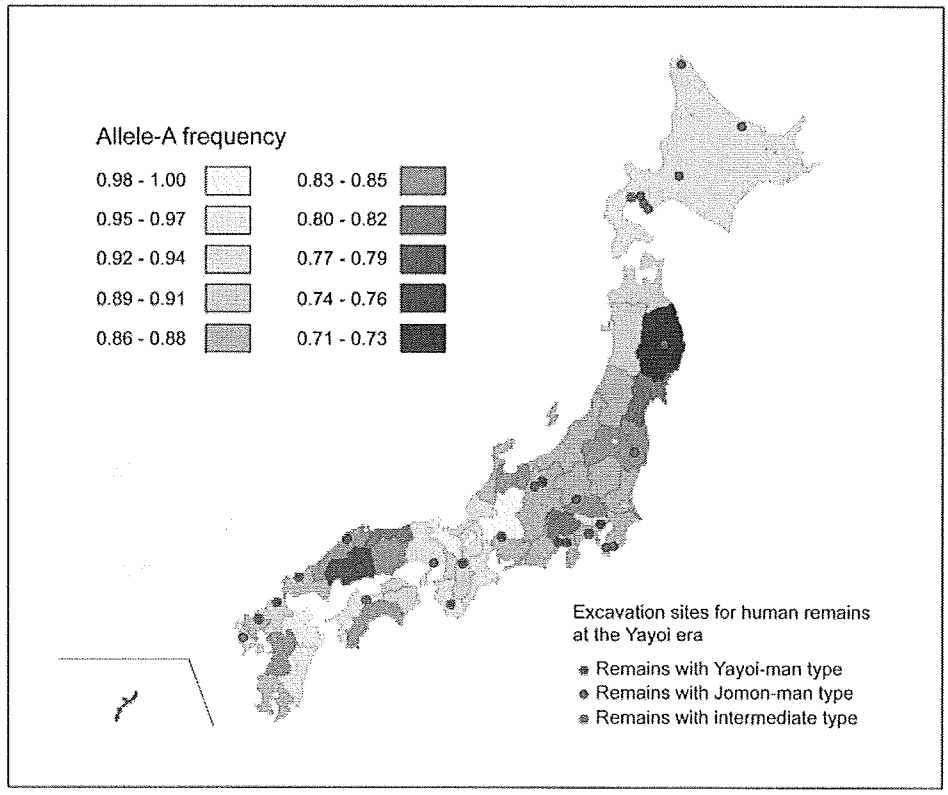

Figure 2 Japanese map of the allele-A (dry-type allele) frequency, merged with excavation sites for human remains at the Yayoi era. The lighter color, the higher frequency of allele A.

archeological sites (data from the National Science Museum, Tokyo, http://shinkan.kahaku.go.jp/index_jp.jsp), where Jomon (blue circle) and Yayoi (red circle) remains have been discovered. Excavation sites for human remains in the Yayoi era are mostly located in Western Japan, whereas those for Jomon-man remains tend to exist in Eastern Japan. It is of great interest that the distribution of Yayoi dig sites overlaps with the areas of higher allele-A frequencies even in the present time, and *vice versa* for Jomon dig sites with lower frequencies, although no statistical difference (P=8980, Mann–Whitney's U-test) was observed in allele frequency between the two integrated areas of prefectures where the two groups of dig-sites were discovered, respectively. Considering the dual-structure model, the overlapping distribution might reflect incomplete mixtures between the two populations with or without wet earwax during the past 3000 years. This argument is supported by the most recent finding on the genetic and anthropological structure of the modern Japanese population.[16] By genotyping at about 140 000 SNP loci among 7001 Japanese, Yamaguchi-Kabata *et al.*[16] found that the Japanese population consists mainly of two clusters, the Ryukyu (Okinawa) and the Hondo (main islands) clusters, which can be characterized by SNP genotypes at the hair thickness gene (*EDAR*) and the earwax gene (*ABCC11*) loci. Earlier studies on carrier rates of adult T-cell leukemia virus (HTLV-1) in the Japanese population showed a geographical distribution similar to that of the wet earwax in this study, and suggested that the characteristics of the native Japanese (the Jomon man) tended to still remain in habitants in Southwestern Kyushu and Southern Shikoku districts.[17,18]

A belt showing high allele-A frequency (lighter color) in Western Japan (Figure 2) may reflect the hypothetical route of the Yayoi people's migration within Japan's islands. They may have been continually coming over through the Korean Peninsula or Southern China first to Northern Kyushu since thousands of years, and migrated along the Seto Inland Sea toward the Kinki area, moving to other areas along the Japan Sea and Pacific Ocean, and finally reaching Northeast Japan. The reasons why the Hiroshima and Aomori prefectures show considerably low and high allele-A frequencies, respectively, remain unknown. Although small sample size and/or high-school selection bias cannot be ruled out, it might suggest an alternative route through Shikoku or a sea route by the Black Current of Pacific Ocean from Eastern Kyushu to Kinki district and far to the north.

In conclusion, a Japanese map of the earwax gene frequency was made by this SSH study. It may provide another line of evidence that suggests a possible route of the Yayoi-man's peopling in Japan. The SSH Consortium strongly hopes that high-school pupils will learn in the near future the achievement of this interdisciplinary study between humanity (Japanese history) and science (genetics) accomplished by the pupils themselves.

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APPENDIX

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