

were calculated using the software FBAT (Horvath et al., 2001; Rabinowitz and Laird, 2000).

Bioethics Approval

Committee approval was obtained from both the ethical committee at Toyohashi Municipal Hospital and Aichi-Gakuin University to obtain whole blood from the patient, his parents, and his paternal grandparents after signed informed consent. For the association analysis, patients were identified at the University of Iowa Hospital and Clinics and in the Philippines through Operation Smile (Murray et al., 1997). Blood samples were obtained with informed consent following institutional review board (IRB) approval in the United States (University of Iowa, IRB Committee) and in the Philippines (Hope Foundation, Bacolod City, Negros, Philippines).

RESULTS

Identification of the Translocation Breakpoint

We found that two clones, RP11-73G18 (164,488 base pairs [bp]) and RP11-999I23 (160,162 bp), included the breakpoint region, judging from a hybridization pattern in which FISH signals were detected simultaneously on the normal chromosome 9 as well as both der(9) and der(17) chromosomes (Fig. 3a). The breakpoint on chromosome 17 was covered by two clones, RP11-770A19 (194,408 bp) and RP11-799L9 (178,732 bp), according to the hybridization pattern in which FISH signals were detected simultaneously on the normal chromosome 17 as well as both der(9) and der(17) chromosomes (Fig. 3b).

To examine sequences of the translocation breakpoints, we tried PCR amplification involving the breakpoint region (Fig. 4a). A primer set of F2 (5'-TCTTAAC-TACCTGTTGCTGCCGTTCCCTG-3') derived from chromosome 9 and R1 (5'-TGGGCTGAGATTGTGG-TAACCGTAGGAAAG-3') derived from chromosome 17 successfully amplified a DNA fragment of approximately 3 kbp in length. The PCR product amplified by 9f1 (5'-GTGTATGATACCATAACAATTAC-3') and 17r1 (5'-GCACTGAGCAAACAGGAAGTG-3') was sequenced after subcloning into the pCR2.1-TOPO vector. The nucleotide sequence of this PCR product demonstrated that the breakpoint of chromosome 9 lies in intron 1 of *SLC31A1* (solute carrier family 31 member 1; GenBank accession number NM001859, between nucleotides 113,072,186 and 113,072,187) according to the UCSC database (hg17). The breakpoint on chromosome 17 is assigned to the 5'UTR of chemokine ligand 2 (*CCL2*; GenBank accession number MN002982, between nucleotides 29,568,964 and 29,568,965). These three exons also form exons 4, 5, and 6 of a predicted six-exon gene of which *CCL2* may be one isoform, according to the Ensembl

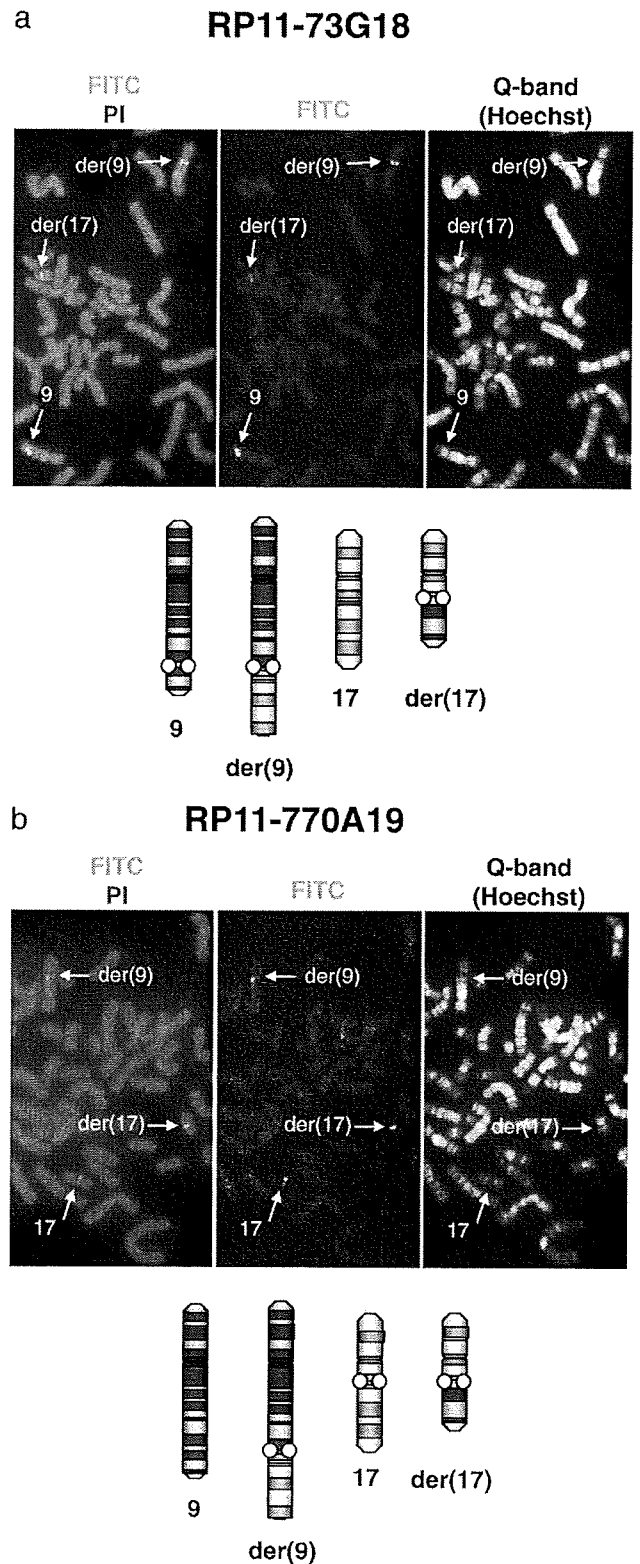


FIGURE 3 Molecular cytogenetic characterization of the t(9;17)(q32;q12). Each left panel shows FISH signals (FITC) on propidium iodide-stained chromosomes (PI). Each right panel exhibits Q-banded chromosomes (Hoechst). The RP11-73G18 clone simultaneously hybridized to normal 9, der(9), and der(17) chromosomes. The RP11-770A19 clone simultaneously hybridized to normal 17, der(9), and der(17) chromosomes. Bottom panels show schematic diagrams of FISH results.

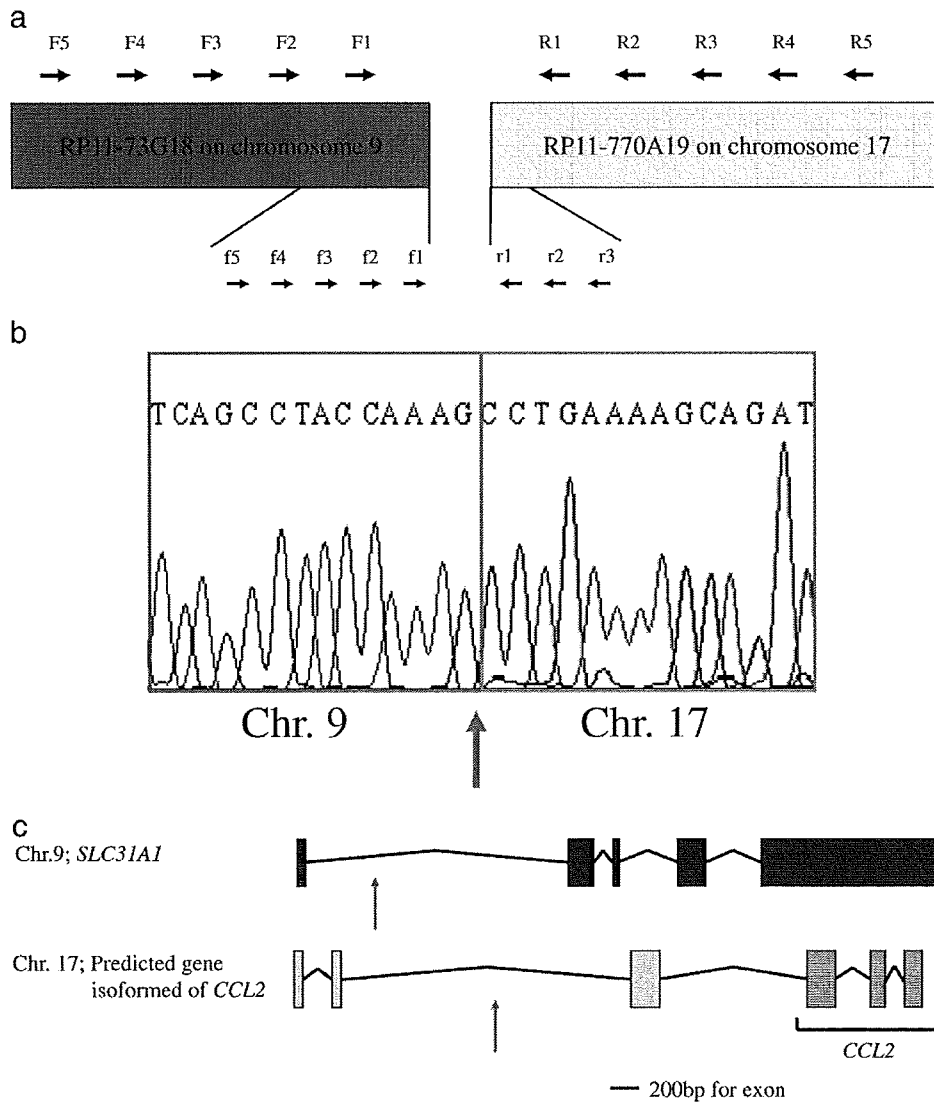


FIGURE 4 Fine mapping of the breakpoints. **a:** Primers used for long-range PCR. Forward and reverse primers were designed every 5 kbp of RP11-73G18 on chromosome 9 and RP11-770A19 on chromosome 17, respectively (arrows in upper). Additional primers flanked regions of the breakpoint (arrows in lower). **b:** Identification of the breakpoint on der(9), using a PCR product with a primer set of 9f1 and 17r1. **c:** The breakpoint on chromosome 9 is located between exons 1 and 2 of *SLC31A1*. The breakpoint on chromosome 17 is located between exons 2 and 3 of a predicted gene, according to the Ensembl database. Arrows indicate the positions of the breakpoints.

database (Transcript ID; GENSCAN00000016010). The function of this predicted gene is unknown. The translocation examined in this study shows no gain or loss of nucleotides at the breakpoint, confirming a perfectly balanced reciprocal translocation in translocation in the proband, his father, and his paternal grandmother (Fig. 4b and 4c).

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

In order to determine whether genes at or near the breakpoint play a role in CL/P, we screened for mutations in three candidate genes: *SCL31A1* and *BSPRY* on

chromosome 9 and the predicted gene that includes exons of *CCL2* on chromosome 17. Although *BSPRY* is located approximately 120 kbp downstream from the breakpoint, we considered it a plausible candidate because it is induced by the fibroblast growth factor receptor (*FGFR*) pathway (Welsh et al., 2007) and impaired FGF signaling has been found to be associated with 3% to 5% of CL/P (Riley et al., 2007). In addition, variants in a related gene, *SPRY2*, that also has a sprouty domain, already have been identified in CL/P (Vieira et al., 2005). We hypothesized that disruption of regulatory elements of *BSPRY* might lead to a cleft phenotype.

Table 1 shows nucleotide variants found in *BSPRY* and the predicted gene that include *CCL2*. In *BSPRY*, two

TABLE 1 Polymorphisms Found (Entries in the Table are Numbers of Individuals With Homozygous Common Allele/Heterozygous Rare Allele/Homozygous Rare Allele)

Variant (Position*)	Filipino Genotyping Distributions			White Genotyping Distributions			Comment
	Case (AF)†	Control (AF)	p Value	Case (AF)	Control (AF)	p Value	
<i>BSPRY</i>							
new (113202320)	62/0/0 (1.000)	68/5/0 (0.966)	.012	62/0/0 (1.000)	55/0/0 (1.000)	~.0	
rs752757 (113202508)	41/19/2 (0.814)	33/32/6 (0.690)	.058	55/22/2 (0.835)	25/7/1 (0.864)	0.756	
new (113202593)	49/2/0 (0.980)	65/3/0 (0.978)	.895	75/1/0 (0.993)	25/0/0 (1.000)	.449	
rs2296074 (113210279)	45/23/1 (0.819)	33/18/0 (0.825)	.565	42/4/0 (0.957)	33/6/0 (0.923)	.341	
Predicted gene (GENSCAN00000016010) containing <i>CCL2</i>							
rs4795884 (29527481)	52/32/6 (0.756)	42/36/1 (0.747)	.103	17/47/17 (0.500)	24/40/19 (0.530)	.396	
new (29527634)	82/0/0 (1.000)	79/0/0 (1.000)	~.0	74/6/0 (0.963)	57/4/0 (0.967)	.828	R15C‡
rs4795885 (29527655)	83/2/0 (0.988)	79/0/0 (1.000)	.103	53/23/3 (0.816)	38/20/3 (0.787)	.830	
new (29540781)	48/30/4 (0.768)	16/12/4 (0.688)	.364	60/19/3 (0.848)	53/25/2 (0.819)	.489	M33T‡
new (29540817)	79/6/0 (0.965)	69/0/0 (1.000)	.007	57/21/4 (0.823)	53/29/1 (0.813)	.198	N45S‡
new (29540841)	86/0/0 (1.000)	69/0/0 (1.000)	~.0	81/1/0 (0.994)	84/0/0 (1.000)	.234	T53M‡
new (29541048)	86/0/0 (1.000)	69/0/0 (1.000)	~.0	69/11/0 (0.931)	75/7/1 (0.981)	.290	
rs1860188 (29602669)	31/21/3 (0.755)	35/21/2 (0.784)	.833	49/12/2 (0.873)	22/13/0 (0.814)	.073	
new (29602875)	54/4/1 (0.949)	47/5/0 (0.952)	.463	63/0/0 (1.000)	73/0/0 (1.000)	~.0	
rs28730833 (29606607)	64/1/0 (0.992)	80/0/0 (1.000)	.204	56/0/0 (1.000)	80/0/0 (1.000)	~.0	
rs4586 (29607382)	10/19/8 (0.527)	11/27/14 (0.471)	.755	20/30/5 (0.636)	5/9/4 (0.528)	.363	

* Position according to UCSC database (hg17).

† AF = allele frequency.

‡ Amino acid change in predicted gene containing *CCL2* according to the Ensembl database (Transcript ID; GENSCAN00000016010).

common polymorphisms (rs752757 and rs2296074) and two new variants (positions 113202320 and 113202593) were detected. One variant (position 113202320) shows a significant *p* value (.012) in the Filipino CL/P population compared with matched controls. In the predicted gene containing *CCL2*, six new variants were detected. Four variants (positions 29527634, 29540781, 29540817, and 29540841) possibly result in a change of amino acid, R15C, M33T, N45S, and T53M, respectively, according to the Ensembl database (Transcript ID; GENSCAN00000016010). R15C, N45S, and T53M were not found in Filipino controls, and T53M was not found in either Filipino or white controls. Five known variants were also identified (rs4795884, rs4795885, rs1860188, rs28730833, and rs4586). The variant at position 29540817 was present with a significant *p* value (.007) in an affected Filipino population compared with control population. No variants were identified in *SLC31A1* (data not shown).

Haplotype Analysis in Markers Adjacent to the Translocation Breakpoint

Although no statistically significant evidence of transmission distortion was seen for either SNP individually, there was statistically significant evidence of transmission distortion for haplotype h2-F9 (C-G-T-T; bi-allelic *p* value = .017) and haplotype h2-C9 (G-A-C-T; biallelic *p* value = .028) on chromosome 9 region (Table 2). We also confirmed statistically significant evidence for haplotype h5-F17 (G-G-T; biallelic *p* value = .042) and haplotype h8-F17 (G-A-A; biallelic *p* value = .017) on chromosome 17 region (Table 3). These *p* values, however, did not reach the level of significance, if we account for the number of tests done and use the conservative Bonferroni correction (0.0071).

DISCUSSION

It has been demonstrated that CL/P is associated with trisomy 9q (Metzke-Heidemann et al., 2004), tetrasomy 9q (Wyandt et al., 2000), and chromosomal translocations involving the 9q region: t(6;9)(p23;q22.3) (Donnai et al., 1992); 46,XX,-22,+der(9)t(9;22)(q22;q11.2) (Pivnick et al., 1990); and 46,X,t(X;9)(p22.1;q32) (Zori et al., 1993). Naritomi et al. (1989) divided trisomy 9q syndrome into four groups (group 1: trisomy for 9q11>q32, group 2: 9q32>qter, group 3: 9q34, and group 4: 9q13/q21>qter) according to the length of the trisomic segment. Of these, the patients in groups 2 and 4 showed cleft palate, suggesting that the 9q32 segment may have important roles for facial development. Genome-wide linkage studies have revealed that the region 9q21 has significant linkage with facial clefting (Marazita et al., 2004). A number of candidate genes for CL/P in this region, 9q21-23, have been reported previously (*ROR2*, *PTCH*, *FOXE1*, and *TGFBR1*) (Ichikawa et al., 2006; Loeyes et al., 2006; Mansilla et al., 2006; Mizuguchi et al., 2004; van Bokhoven et al., 2000; Vieira et al., 2005). Using a positional cloning approach, we demonstrated that *SLC31A1* on chromosome 9 was disrupted within its intron. The *SLC31A1* encodes a copper transporter and is itself not a strong candidate for involvement in facial development; although, unbalanced copper regulation can disturb cell metabolism (Lee et al., 2001). However, it is increasingly recognized that point mutations in regulatory elements located far from gene structural elements can be damaging. For example, mutations located up to ~1 Mbp from the gene *SHH* are capable of causing congenital abnormalities (Gurnett et al., 2007; Lettice et al., 2003) and campomelic dysplasia has resulted from a balanced translocation breakpoint 1.3 Mbp

TABLE 2 Results of Haplotype-based Transmission Disequilibrium Test for Single-Nucleotide Polymorphisms (SNPs) Around the Breakpoint on Chromosome 9

Filipino Haplotype	SNP				Estimated Frequency	p Values (Biallelic)
	a*	b	c	d		
h1-F9	C	A	T	T	0.282	.456 (-)
h2-F9	C	G	T	T	0.246	.017 (+)
h3-F9	C	G	C	T	0.206	.095 (-)
h4-F9	C	A	T	C	0.053	.809 (+)
h5-F9	C	G	T	C	0.052	.892 (+)
h6-F9	G	A	T	T	0.047	.638 (-)
h7-F9	C	G	C	C	0.031	.093 (-)
h8-F9	G	G	C	T	0.027	.367 (+)
h9-F9	C	A	C	T	0.015	.594 (+)
h10-F9	G	G	T	T	0.013	.815 (+)
h11-F9	G	A	T	C	0.010	.956 (-)
h12-F9	G	G	C	C	0.010	.476 (+)

White Haplotype	SNP				Estimated Frequency	p Values (Biallelic)
	a	b	c	d		
h1-C9	G	A	T	T	0.137	.111 (-)
h2-C9	G	A	C	T	0.121	.028 (-)
h3-C9	G	A	T	C	0.110	.441 (+)
h4-C9	C	A	C	T	0.102	.342 (-)
h5-C9	G	A	C	C	0.087	.224 (+)
h6-C9	C	A	T	T	0.085	.122 (+)
h7-C9	G	G	T	C	0.065	.103 (+)
h8-C9	G	G	T	T	0.062	.624 (+)
h9-C9	G	G	C	T	0.059	.246 (+)
h10-C9	C	G	T	T	0.056	.198 (-)
h11-C9	C	G	C	T	0.039	.819 (+)

* a = rs1330692 C/G; b = rs3750534 A/G; c = rs4596714 C/T; d = rs974230 C/T.

downstream of the *SOX9* gene (Velagaleti et al., 2005). In the present report, although we did not detect any mutations in the *SLC31A1* gene itself, we did detect a significant *p* value for CL/P in the case-control study of a strong candidate for craniofacial development, the *BSPRY* gene, which is located 120 kbp downstream from the breakpoint (Table 1). Thus

the translocation break may disrupt not the gene itself but regulatory regions. Moreover, there was significant evidence of transmission distortion for haplotypes on chromosome 9 (Table 2). In summary, it is possible that this region of chromosome 9q contains one or more genes playing a role in facial development.

TABLE 3 Results of Haplotype-Based Transmission Disequilibrium Test for Single-Nucleotide Polymorphisms (SNPs) Around the Breakpoint on Chromosome 17

Filipino Haplotype	SNP			Estimated Frequency	p Values (Biallelic)
	e*	f	g		
h1-F17	A	G	T	0.322	.451 (+)
h2-F17	A	A	T	0.316	.991 (+)
h3-F17	A	A	A	0.103	.782 (+)
h4-F17	A	G	A	0.102	.973 (+)
h5-F17	G	G	T	0.065	.042 (-)
h6-F17	G	A	T	0.054	.450 (-)
h7-F17	G	G	A	0.025	.765 (-)
h8-F17	G	A	A	0.013	.017 (+)

White Haplotype	SNP			Estimated Frequency	p Values (Biallelic)
	e	f	g		
h1-C17	A	A	A	0.180	.275 (+)
h2-C17	A	A	T	0.164	.592 (+)
h3-C17	G	A	T	0.136	.597 (+)
h4-C17	G	A	A	0.120	.684 (-)
h5-C17	A	G	A	0.115	.899 (+)
h6-C17	G	G	T	0.112	.547 (-)
h7-C17	A	G	T	0.099	.534 (-)
h8-C17	G	G	A	0.075	.161 (-)

* e = rs16561 A/G; f = rs1029719 A/G; g = rs725276 A/T.

It is also possible that facial development may be influenced by genes on chromosome 17q. Case reports of chromosome translocations involving 17q24 (Czako et al., 2004; Luke et al., 1992), 17q25 (Bridge et al., 1985), 17q21.1 (Martinet et al., 2006), and 17q23.3 (Stalker et al., 2001) are associated with CL/P. Additively, genome-wide linkage analysis also shows significant association of the 17q21 region to the occurrence of CL/P (Marazita et al., 2004). Three candidate genes for CL/P in this region, 17q23–25, have been described (*SEPT9*, *MKSI*, and *GAA*) (Huie et al., 1999; Jeannet et al., 2001; Paavola et al., 1995). In addition, *RARA*, which is located approximately 6 Mbp downstream from the breakpoint studied here, is associated with CL/P (Chenevix-Trench et al., 1992). In this study we identified a predicted gene containing *CCL2* in the 17q breakpoint region. *CCL2* (monocyte chemotactic protein-1) is a member of the small inducible gene family and plays a role in the recruitment of monocytes to sites of injury and infection and so is not an obvious candidate for CL/P. We did, however, observe multiple amino acid variants in the predicted gene. One of them, a heterozygous variant in position 29540817 (possibly N45S), showed a significant *p* value in the Filipino CL/P population compared with the control population (Table 1). Moreover, there was statistically significant evidence of transmission distortion for two haplotypes on chromosome 17 in Filipinos but not in the white population (Table 3). These data suggest that the participation of this region in facial development may vary among different populations, and these results warrant an extension of these studies to larger numbers for confirmation.

Although three generations were examined here segregating the same chromosome translocation, only one individual was clearly affected with cleft lip and palate. CL/P is commonly nonpenetrant even in disorders caused by single gene mutations. Mutations in *IRF6* cause the autosomal dominant van der Woude syndrome, but there is only 70% penetrance for the CL/P phenotype (Burdick, 1986; Zuccherro et al., 2004). Mutations in *MSX1* and several of the FGF and FGFR genes may also cause CL/P, but again only a subset of individuals with the mutation have a cleft phenotype (van den Boogaard et al., 2000). Isolated clefts are complex traits, not single-gene Mendelian disorders. They are likely caused by several interacting genes, each with small effects. In our case, the presumed loss of 50% of gene product may be the tipping point in an individual already having several other of the predisposing alleles/genes that may be absent in other family members with the translocation, hence their unaffected status. Clearly, future work is required to develop a better understanding of penetrance in CL/P.

In summary, we report an extensive study of candidate genes for CL/P based on a family with CL/P and segregating a balanced chromosome translocation t(9;17)(q32;q12). Analysis of the translocation shows disruption of *SLC31A1* on chromosome 9 and a predicted gene that includes *CCL2* on

chromosome 17. It is intriguing that *SLC31A1* lies close to *BSPRY*, an excellent candidate gene for CL/P, and the results of a case-control study support a role for *BSPRY* in CL/P. In the aggregate, these data may provide additional support for an intensive search for genes/mutations and also for micro-deletions in the 9q/17q region that would play a role in CL/P.

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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 Consortium¹

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, Kishi³ first described the different nature of earwax between the Japanese and Europeans. Adachi⁴ 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. Matsunaga⁵ 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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¹Members of the SSH Consortium (asterisk) and other high schools/universities that participated in the SSH Consortium study are listed in the Appendix.
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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 CAGTGTACTCAGGCCAG for hydrolyzing VIC-labeled TaqMan MGB wet probe and FAM-labeled dry probe, respectively; and CTCTGGGCATCTGCTTCTG 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				Allele-A frequency ± 95% confidence interval
	AA	GA	GG	Total	
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

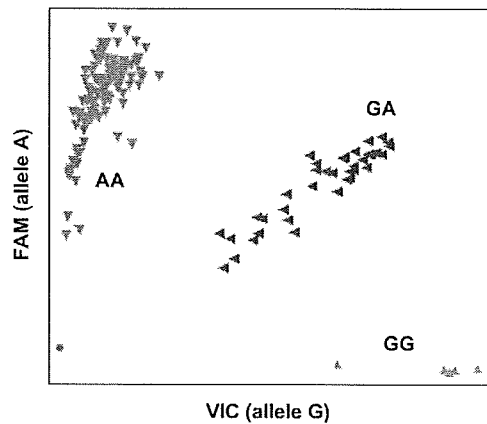


Figure 1 Three different genotypes at the rs17822931 locus by the TaqMan PCR method. 'AA', 'GA' and 'GG' are AA homozygotes (phenotypically dry type) GA heterozygotes (wet type) and GG homozygotes (wet type), respectively.

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).

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

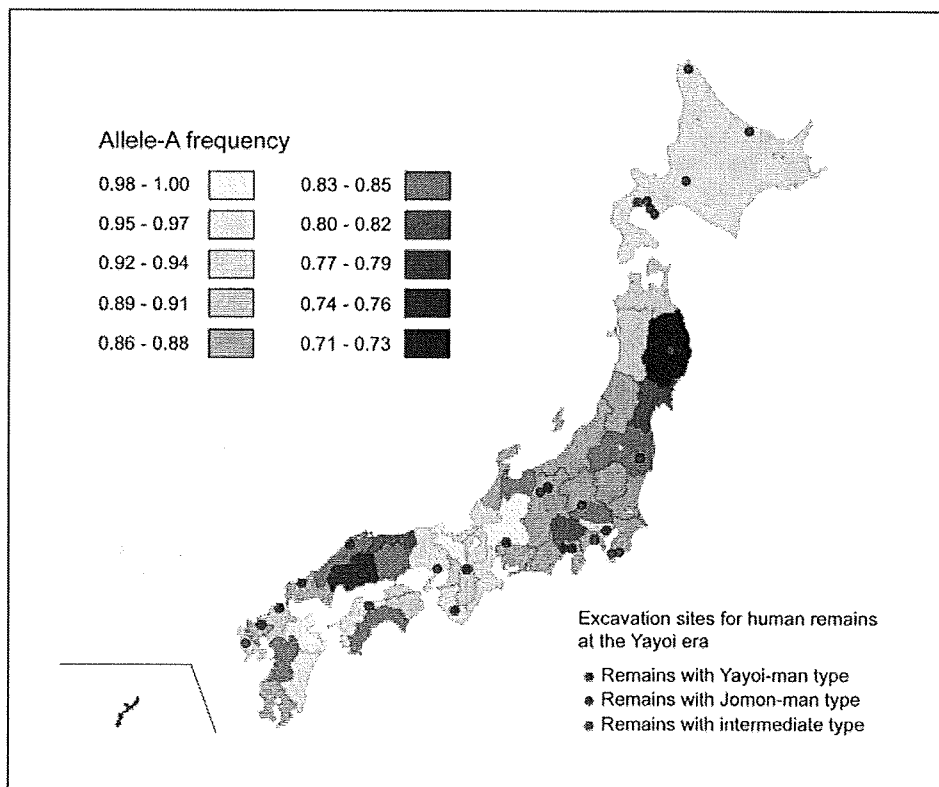


Figure 2 Japanese map of the allele-A (dry-type allele) frequency, merged with excavation sites for human remains at the Yayoi era. The lighter color, the higher frequency of allele A.

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 inhabitants 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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Search for Genomic Alterations in Monozygotic Twins Discordant for Cleft Lip and/or Palate

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Phenotypically discordant monozygotic twins offer the possibility of gene discovery through delineation of molecular abnormalities in one member of the twin pair. One proposed mechanism of discordance is postzygotically occurring genomic alterations resulting from mitotic recombination and other somatic changes. Detection of altered genomic fragments can reveal candidate gene loci that can be verified through additional analyses. We investigated this hypothesis using array comparative genomic hybridization; the 50K and 250K Affymetrix GeneChip® SNP arrays and an Illumina custom array consisting of 1,536 SNPs, to scan for genomic alterations in a sample of monozygotic twin pairs with discordant cleft lip and/or palate phenotypes. Paired analysis for deletions, amplifications and loss of heterozygosity, along with sequence verification of SNPs with discordant genotype calls did not reveal any genomic discordance between twin pairs in lymphocyte DNA samples. Our results demonstrate that postzygotic genomic alterations are not a common cause of monozygotic twin discordance for isolated cleft lip and/or palate. However, rare or balanced genomic alterations, tissue-specific events and small aberrations beyond the detection level of our experimental approach cannot be ruled out. The stability of genomes we observed in our study samples also suggests that detection of discordant events in other monozygotic twin pairs would be remarkable and of potential disease significance.

Keywords: monozygotic twins, discordant, cleft lip and palate, genome-wide

Classical twin research studies comparing disease concordance rates between monozygotic (MZ) and dizygotic (DZ) twins have been extensively applied to estimate the contribution of genetic and environmental factors to many complex traits (Boomsma et al., 2002). MZ twin concordance for common diseases and traits rarely reaches 100%, an observation that is

often attributed to differential environmental exposures (Wong et al., 2005). However, there are several molecular mechanisms that could underlie phenotypic discordance between MZ twins, such as *de novo* somatic mutations (Kondo et al., 2002), chromosomal anomalies (Gilbert et al., 2002), skewed X chromosome inactivation (De Gregorio et al., 2005), imprinting defects (Weksberg et al., 2002) and differential gene expression (Mak et al., 2004). The identification of molecular genetic differences between discordant MZ twins suggests that the utility of twin studies could be extended beyond heritability studies to gene discovery.

Postzygotic mitotic recombination and other somatic events such as deletions, nondisjunction, gene conversion, mobile genetic elements, fragile sites and repeat expansions have been proposed as a cause of MZ twin discordance (Cote & Gyftodimou, 1991; Kastern & Kryspin-Sorensen, 1988). Occurrence of somatic genomic changes in the early embryo can have significant implications for the phenotypes of the twins and the twinning process. If a genomic alteration occurs prior to twinning, unequal allocation of cells with the lesion to the two embryos could account for discordant phenotypes, with evidence of mosaicism, while the segregation of two genetically different populations of cells may trigger the twinning process, resulting in co-twins with discordant phenotypes (Machin, 1996). Since detection of chromosomal abnormalities relies on the comparison of a test genome with a reference genome sequence, the identical genomes of MZ twins ensure that any genomic differences can be established with confidence and

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accuracy. This approach is particularly advantageous for delineating disease causing copy number variants that could arise in one member of the twin pair. Any genomic mismatches between the twins' genomes could be revealed as amplifications, deletions or loss of heterozygosity. Recent identification of novel cleft lip and/or palate (CLP) candidate genes by genome-wide array-CGH highlights the impact of genomic alterations in the etiology of this complex disorder (Osoegawa et al., 2008).

CLP occurs in approximately 1 out of 700 births worldwide and has a significant clinical and economic impact. Approximately 40% of MZ twins are discordant for the disease phenotype (Christensen & Fogh-Andersen, 1993a). Genetic factors contributing to disease etiology include *IRF6* (Zuccherro et al., 2004); *MSX1* (Jezewski et al., 2003); *RYK* (Watanabe et al., 2006) and genes in the *FGF* pathway (Riley et al., 2007). Additionally, nongenetic factors such as maternal cigarette smoking and nutrition also play a role in CLP etiology (Jugessur & Murray, 2005).

The complex nature of CLP complicates traditional mapping approaches such as linkage and association,

which rely on population-matched or family-based controls to determine if a sequence variant is disease-causing. Based on identical genetic backgrounds, the unaffected twin in a discordant pair provides a well-matched control for studying a complex disease such as CLP thereby eliminating the need for external controls. We have previously reported our model using MZ twins discordant for CLP in gene discovery (Mansilla et al., 2005). In this report, we describe our search for genomic alterations in MZ twins discordant for CLP using array CGH and high-density SNP genotyping arrays.

Materials and Methods

Study Subjects

A summary of the discordant MZ twin samples and the respective analyses employed for each twin pair is presented in Table 1. The variability in the methods used to analyze the different samples resulted from multiple stages of the study being conducted over a period of time. All affected individuals were nonsyndromic cases of CLP. We used DNA previously

Table 1

Twin Samples Analyzed in This Study

Twin pair no.	Twin sample data			Analysis method			
	Country	Sex	Phenotype	Array CGH	Affymetrix 50K	Affymetrix 250K	Custom Illumina SNPs
1	P	F	CL+P	✓			✓
2	P	M	CLO	✓	✓		✓
3	P	M	CL+P	✓			✓
4	P	F	CLO	✓		✓	✓
5	P	F	CL+P	✓	✓		✓
6	C	M	CL+P	✓		✓	
7	C	M	CLO			✓	
8	P	F	CL+P			✓	✓
9	B	M	CL+P			✓	
10	B	F	CLO			✓	✓
11	B	M	CLO			✓	✓
12	B	F	CLO			✓	
13*	B	F	CL+P			✓	
14	P	M	CL+P			✓	✓
15	A	F	U/T				✓
16	A	M	U/T				✓
17	A	M	U/T				✓
18	D	M	CLO				✓
19	D	M	CLO				✓
20	D	F	CLO				✓
21	D	F	CLO				✓
22	D	F	CLO				✓
23	D	M	CLO				✓
24	D	F	CL+P				✓
25**	D	F	CPO				✓

Note: *Father's DNA sample unavailable; **Triplets; A-Australia; B-Brazil; C-Colombia; D-Denmark; P-Philippines; U/T-Untyped cleft phenotype.

extracted from peripheral blood lymphocytes. Genotyping a set of DNA markers had previously established that the twins were MZ. Informed consent was obtained for all study participants and the institutional review board approved the study.

Array CGH

CGH is a molecular cytogenetic technique for analyzing DNA copy number variations. DNA from a test and reference sample are differentially labeled and hybridized to an array spotted with a genomic representation that allows detection of copy number differences between the two samples at specific genomic locations (Pinkel & Albertson, 2005). For our analysis, Koh-ichiro Yoshiura et al. at Nagasaki University developed an array spotted with 2,173 genomic BAC clones. Samples from six pairs of twins were analyzed: for each pair, the DNA samples were differentially labeled, hybridized, and then scanned. Reverse sample labeling was also performed. The average normalized inter-locus fluorescence ratio (ANILFR) between the affected and unaffected twin samples was calculated. Prior preliminary experiments using five sets of normal/normal control samples had established the thresholds for copy number gain and loss at 0.86 and 1.18 with a standard deviation (*SD*) of 0.06, so the normal ANILFR range was defined as within ± 2 *SD*.

SNP Genotyping Arrays

Large scale SNP genotyping allows detection of allelic imbalances such as loss of heterozygosity (LOH) and copy number changes through hybridization signal intensities. In our analysis, we utilized a custom BeadArray™ platform from Illumina, Inc., (San Diego, CA, USA) and Affymetrix GeneChip® Human Mapping 50K and 250K sets (Santa Clara, CA, USA).

Illumina custom SNP genotyping. The Illumina BeadChip is a platform for performing multiplex gene analyses using oligonucleotides attached to silica beads. Defined SNPs can be chosen for a custom genotyping array based on the interests of the researcher (Steemers & Gunderson, 2007). For our analysis, we

selected 1,536 SNPs representing 388 CLP candidate genes for a single genotyping array. The experiments were carried out at the Center for Inherited Disease Research (CIDR) according to the manufacturer's instructions and included 20 pairs of discordant MZ twins. Data were analyzed using Illumina's BeadStudio v2 genotyping and LOH plus module software that allow detection of chromosomal aberrations and allelic imbalance in paired samples (www.illumina.com).

Affymetrix SNP genotyping. Affymetrix GeneChips employ a whole genome sampling analysis method to genotype thousands of SNPs on synthetic oligonucleotide arrays by allele-specific hybridization. DNA samples were prepared for analysis and hybridization according to the manufacturer's instructions (www.affymetrix.com). We used the GeneChip® Human Mapping 50K *Xba* I (*n* = 2 twin pairs) and the 250K *Nsp* I (*n* = 10 twin pairs) arrays. SNP allele calls were assigned using a Dynamic Model mapping algorithm, a highly accurate genotype calling method that uses a one-sided Wilcoxon signed rank test to provide a confidence score (CS) for each genotype. The CS measures the reliability of a genotype call thus filtering out SNPs with a high error rate as 'no-calls' (Matsuzaki et al., 2004). Gender status for each sample is inferred based on X chromosome heterozygosity. We performed a paired analysis for LOH and copy number changes using the Affymetrix Chromosome Copy Number Analysis Tool 4.0 (CNAT 4.0).

DNA Sequencing

Genotypes generated using the Affymetrix 50K and 250K GeneChips that had discordant allele calls and a confidence score of ≤ 0.05 were identified as candidates for DNA sequencing to verify the SNP genotypes. After ranking by the significant confidence scores, at least 10 SNPs that did not fall within repeat elements were selected for sequencing in each twin pair. DNA samples from the parents were included in the sequencing analysis to verify Mendelian segregation of alleles. PCR reactions were performed on Applied Biosystems Gene Amp PCR System 9700 with 20ng

Table 2

BAC Clones With Significantly High Signal Differences From aCGH

BAC Clone	Locus	Twin pair no.					
		1 ^a	2 ^b	3 ^a	4 ^{a,c}	5 ^{a,b}	6 ^c
GS-98C4	XpYpter			✓	✓	✓	
RP11-89P7	2q32.1 - 2q32.2			✓		✓	
RP11-1145M16	7q11.2					✓	✓
RP11-117N14	8q21.11	✓			✓		
RP11-613G2	11p15.5		✓				✓
RP11-75H6	19p13.3			✓			✓
RP11-17K15	19p13.3		✓			✓	
RP11-19704	19p13.2			✓	✓		

Note: Samples also genotyped with the Illumina genotyping panel^a, the 50K Affymetrix GeneChip^b, or the 250K Affymetrix GeneChip^c.

Table 3
Summary of Affymetrix GeneChips Genotyping Results

50K Affymetrix GeneChip				
Twin pair no.	SNP Call rate (affected/unaffected) average: 91.4%	Genotype concordance average -99.7%	No. of discordant genotypes	Discordant genotypes with ≤ 0.05 confidence score
2	90.7 / 92.7	99.7	180	4
5	91.3 / 91.5	99.8	137	5
250K Affymetrix GeneChip				
Twin pair no.	SNP Call rate Average -91.5%	Genotype concordance Average -98.8%	No. of discordant genotypes	Discordant genotypes with ≤ 0.05 confidence score
4	95.7 / 93.9	99.3	1818	13
6	87.7 / 88.3	98.6	3668	16
7	86.0 / 85.7	98.0	5349	64
8	93.4 / 94.0	99.2	2126	7
9	90.8 / 90.7	98.6	3607	32
10	91.9 / 92.1	98.9	2945	27
11	95.3 / 93.6	99.2	2027	15
12	94.5 / 92.4	99.0	2528	24
13	93.2 / 93.0	99.0	2711	35
14	92.6 / 90.5	98.8	3052	35

DNA and 0.25 units of Biolase (Bioline, Randolph, MA). Sequencing was carried out with the Big Dye™ Terminator cycle sequencing and run on ABI Prism 3730 DNA Analyzer (Applied Biosystems). Sequences were assembled using the Phred-Phrap package and visualized using the Consed program.

Results

Array CGH Results

With 2,173 BAC clones spread across the genome, the aCGH provided a resolution of approximately 1-Mb. Table 2 shows 8 BAC clones with fluorescence ratios that deviated significantly (> 3 SD) in at least two twin pairs. The regions covered by these BACs do not overlap with any known CLP candidate loci, nor contain genes showing high craniofacial expression according to COGENE — the Craniofacial and Oral Gene Expression Network. Genomic region 19p13 looked especially interesting with 3 BACs showing high signal differences, so we genotyped two SNPs of high heterozygosity within each BAC in the twins and the parents, but did not find evidence for allelic imbalance.

Illumina Genotyping Results

The average SNP call rate on the Illumina genotyping panel was approximately 89%. We did not observe any discordant genotypes between pairs of MZ twins. A paired analysis with the Beadstudio software was performed for LOH and copy number changes at a 1 Mb window size. Although the sparse SNP coverage of ~1,500 SNPs greatly limited the power of this analysis, there was no indication of LOH in the twin pairs. A few genomic regions revealed possible copy

number changes for twin pairs 3–6, but the more comprehensive genotype data generated from Affymetrix arrays disproved that observation.

Affymetrix Genotyping Results

Genotyping data and concordance rates for the samples scanned with the Affymetrix GeneChips are summarized in Table 3. The call rate from 58,960 and 262,264 SNPs for the 50K and 250K GeneChips respectively was $> 90\%$ for all samples. The average proportion of genotypes that were concordant between twin pairs was ~99% for both GeneChips. This was comparable to the 98% degree of genotype concordance observed in comparing two independent 250K array scans of the unaffected individual in twin pair no. 6, which revealed high reproducibility of the genotype calls.

Sequencing Results

DNA sequencing was carried out for a total of 107 regions surrounding SNPs that had received discordant genotype calls from the Affymetrix GeneChip analyses within twin pairs. Results revealed 181 SNP genotypes that were concordant between twin pairs (Table 4). Additionally, sequencing of DNA samples from the parents showed consistency with Mendelian inheritance.

Discussion

The advent of genome scanning tools allows comprehensive analysis of chromosomal rearrangements, dependent on the resolution of the experimental approach. The aCGH experiment using genomic

Table 4
Summary of SNPs Genotyped by Sequencing to Confirm Concordance Between Twin Pairs

Twin pair	Region (no. SNPs)													
2	2q23.3 (1)	5q12 (1)	10q21 (1)	16p12.1 (1)										
5	8q24.12 (3)	10q21.1 (1)	12q13.13 (1)	15q26.2 (1)										
4	2q14.3 (1)	3q27.2 (1)	10p15.2 (1)	10q26.2 (1)	13q31.1 (1)	16q23.3 (1)	21q21.1 (2)							
6	1q32.1 (4)	4p15.32 (3)	4q32.3 (1)	5q21.3 (1)	7p12.1 (3)	7q31.32 (2)	8q22.1 (2)	9p24.2 (2)	10q11.23 (1)	10q21.1 (4)	11q24.2 (1)	12q15 (1)	14q31.3 (1)	
7	2q21.3 (1)	5p15.33 (2)	6q14.1 (1)	7p21.1 (1)	12q21.2 (1)	12q21.32 (2)	12q23.2 (2)	16q22.3 (1)	18q12.3 (1)	19q13.32 (4)				
8	2p24.1 (1)	2q21.3 (1)	4p13 (1)	5q21.3 (1)	8q24.22 (3)	11p13 (2)	12q21.33 (1)	21q21.1 (2)	Xq25 (2)					
9	2q13 (2)	2q31.2 (2)	4q31.23 (1)	5p13.3 (1)	5q14.1 (3)	7q21.11 (1)	10q23.31 (3)	11p15.4 (2)	11p14.2 (2)	17q11.2 (1)				
10	1q32.1 (4)	1q32.1 (5)	2p16.1 (3)	3p14.2 (1)	5p13.2 (1)	5q14.3 (3)	5q35.2 (3)	7p21.2 (1)	8q23.2 (1)	14q21.3 (2)				
11	1q25.3 (1)	2q34 (1)	4q22.3 (3)	5q12.1 (3)	7p21.2 (1)	7q21.3 (2)	11p14.1 (1)	12p12.3 (1)	12q12 (1)	Xq28 (2)				
12	1p36.12 (1)	1q44 (3)	2p24.3 (5)	4p16.3 (1)	6q13 (3)	6q16.1 (1)	7p15.1 (1)	10q21.3 (1)	10q26.11 (1)	18q12.3 (1)				
13	1p12 (1)	1q24.2 (4)	3q23 (2)	3q25.33 (1)	6p25.3 (1)	6q16.3 (2)	9p21.3 (1)	10p15.1 (1)	16q12.2 (2)	17q22 (1)				
14	3p12.3 (1)	3q13.33 (1)	4p12 (2)	6q16.1 (1)	8p23.2 (2)	8q23.1 (2)	8q23.2 (1)	10p12.32 (1)	10q21.3 (2)	11p11.2 (1)				

BACs provided extensive coverage of the genome at a resolution of ~1 Mb, so any smaller chromosomal aberrations would not be detected. SNP arrays not only provide genotypes for thousands of SNPs, but can also be used to detect copy number changes based on hybridization signal intensities. Genotype data is useful for detecting loss of heterozygosity in chromosomal regions with deletions or uniparental disomy. At an average inter-marker distance of ~60 kb and ~12 kb for the 50K and 250K GeneChips respectively, and additional genotyping through the Illumina SNP panel, our analysis provide sufficient resolution to detect submicroscopic structural variants that are defined in the range of ~10 kb to 3 Mb (Feuk et al., 2006). Several samples were interrogated using more than one method (table 1), thus allowing data comparison and verification.

Our study using aCGH and genotyping arrays did not reveal any genomic alterations within MZ twins discordant for nonsyndromic CLP. Genomic alterations could be confined to specific tissues depending on the timing of the mutational event, resulting in mosaicism. Since acquisition of DNA samples from lip and palatal tissues is difficult, our analysis was limited to analysis of DNA samples collected from peripheral blood lymphocytes, which may not accurately represent the target tissues affected in CLP. Additionally, our experimental approaches were incapable of detecting balanced variants such as those resulting from inversions and translocations, and chromosomal aber-

rations that involve regions with only homozygous alleles. Routine karyotyping which can generally detect such chromosomal abnormalities could not be performed due to unavailability of living cells from a blood sample.

We are unaware of definitive reports of postzygotic genomic rearrangements underlying MZ twin discordance. A recent report of copy number variants arising between MZ twins during somatic development is consistent with our rationale for using discordant MZ twins in disease gene identification (Bruder et al., 2008). Changes in the somatic genome are well recognized as a source of diversity within the immunoglobulin and T-receptor genes (Kastern & Kryspin-Sorensen, 1988). In disease states, rearrangements can induce a phenotype by directly interrupting a gene sequence, altering gene dosage, or gene expression through position effects (Lupski & Stankiewicz, 2005). Mitotic recombination is especially relevant in tumor development, since it can lead to the expression of recessive tumor suppressor genes and/or amplification of protooncogenes (Gupta et al., 1997). Analysis of MZ twins concordant for cancer can reveal DNA rearrangements that are common to both twins as potential candidates for susceptibility loci (el-Rifai et al., 1999). In addition to oncogenesis, somatic mutations mediated through LOH can potentially contribute to other biological processes such as aging (Grist et al., 1992).

Phenotypic discordance is a common observation in complex genetic diseases, and a postzygotic somatic change is only one of several proposed mechanisms for discordance. Since CLP is a congenital defect, non-genetic intrauterine environmental factors such as unequal cell allocation at twinning and disproportionate placental blood supply may contribute to discordance (Gringras & Chen, 2001). If such developmental influences alone were responsible for MZ twin discordance, then one would expect an excess of CLP in MZ twins compared to singletons. This is not the case as studies in Denmark have demonstrated no significant difference in prevalence of CLP in twins versus singletons (Christensen & Fogh-Andersen, 1993a; 1993b). The absence of genomic differences between MZ twins underscores their use as matched case-controls particularly in studies exploring the environmental component in complex traits. With the availability of array-based techniques to simultaneously scan the whole genome, it is now possible to map genomic alterations at a high resolution and additional experimental tools such as quantitative PCR and multiplex ligation-dependent probe amplification (MLPA) can be applied to independently confirm any observed genomic alterations. This straightforward approach can be applied to other cases of discordant MZ twins where identification of genomic alterations can reveal potential candidate genes, or provide evidence of a gene's involvement in disease etiology.

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Original Article

Human Papillomavirus DNA in Plasma of Patients with HPV16 DNA-positive Uterine Cervical Cancer

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Objectives: The squamous cell carcinoma antigen is considered the most accurate serologic tumor marker for uterine cervical carcinoma. However, serum squamous cell carcinoma antigen levels were found to correlate significantly with clinical severity of atopic dermatitis and chronic renal failure. The present study was conducted in patients with human papillomavirus 16 DNA-positive uterine cervical cancer to determine the plasma level of human papillomavirus 16 DNA and the diagnostic values of plasma human papillomavirus DNA in these patients.

Methods: Forty-three human papillomavirus 16-positive patients with cervical intraepithelial neoplasia or uterine cervical squamous cell carcinoma were recruited in this study. The diagnosis was cervical cancer in 20 patients, high-grade squamous intraepithelial lesions in 21, low-grade squamous intraepithelial lesions in 1 and negative for intraepithelial lesion or malignancy in 3 patients. Before any treatment, blood samples were collected from all patients. For analysis of human papillomavirus DNA in plasma of patients with cervical cancer, quantitative polymerase chain reaction fluorescent assay for human papillomavirus 16 was performed using human papillomavirus 16 primers and SYBR Green dye using the LightCycler 480 SW1.5 apparatus.

Results: Plasma human papillomavirus 16 DNA was detected in only 30.0% of the patients with human papillomavirus 16-positive cervical cancer and in none of normal controls. The copy number of plasma human papillomavirus 16 DNA was higher in patients with invasive cancer than in those with cervical intraepithelial neoplasia (CIN3), micro-invasive cancer and in normal individuals.

Conclusions: These results indicated that the plasma human papillomavirus DNA level could be potentially used as a marker of low-invasive cervical cancer tumors in patients with normal squamous cell carcinoma antigen levels before treatment.

Key words: gynecol-basic – genetics-cancer genetics – diagnosis

INTRODUCTION

The squamous cell carcinoma antigen (SCCA), an SCC tumor-associated protein, was first discovered in uterine cervical SCC by Kato and Torigoe (1) in 1977. SCCA is

expressed not only in SCC but also in normal squamous epithelium (2) and considered the most accurate serological marker of uterine cervical carcinoma. However, serum levels of SCCA have been reported also to correlate with the

clinical severity of atopic dermatitis, chronic hypertension and chronic renal failure (3).

Epidemiological studies have provided data on the incidence of human papillomavirus (HPV) infection and the risk factors for HPV infection and genital precancerous lesions (4–6). The long duration of HPV infection is attributed to the ability of the virus to subvert innate immune responses (7,8). Persistent infection with HPV confers a strong risk for the development of subsequent neoplasia (9,10). Several groups have examined the prevalence of HPV DNA in plasma of cervical cancer patients, although there is discrepancy in the results of these studies (11–14). The different prevalence rates could be due to the different populations examined, different HPV types and different methods used for the detection.

The present study was designed to determine the prevalence of HPV16 DNA in plasma of patients with HPV16 DNA-positive uterine cervical cancer and its diagnostic and prognostic value in these patients.

PATIENTS AND METHODS

PATIENTS AND SAMPLE COLLECTION

This study was approved by the Human Ethics Committee of Nagasaki University Hospital. Forty-three HPV16-positive patients with cervical intraepithelial neoplasia (CIN) or uterine cervical SCC were recruited in this study. All patients were examined and/or treated at Nagasaki University Hospital between December 2007 and June 2008. Each patient underwent pelvic examination followed by conventional cervical cytology and determination of the serum SCCA level (see below). After obtaining informed consent, specimens for HPV typing were harvested (SurePath and CytoRoch, MBL) and evaluated for HPV DNA by polymerase chain reaction (PCR).

Table 1 shows the stage of cervical cancer and treatment modalities. The diagnosis was cervical cancer in 20 patients, high-grade squamous intraepithelial lesions in 21, low-grade squamous intraepithelial lesions in 1 and negative for intraepithelial lesion or malignancy (NILM) in 3 patients. The stage of cervical cancer was CC Ia1 in 4 patients, CC Ia2 in 1, CC Ib1 in 2, CC Ib2 in 2, CC IIa in 4, CC IIb in 4, CC IIIb in 1, CC IVa in 1 and CC IVb in 1, according to the International Federation of Gynecology and Obstetrics (FIGO) staging system. We also recruited 20 normal individuals who had no history of cervical dysplasia or neoplasia, as negative controls. Subjects of the control group were confirmed by cervical cytology to be free of cancer or intraepithelial lesions and by hybrid Capture II test to be HPV DNA-negative. Before any treatment, 7 ml blood samples were collected from all patients and placed immediately into tubes containing ethylenediaminetetraacetic acid.

SCCA ASSAY

Serum SCCA levels were assayed by radioimmunoassay (RIA, SRL, Tokyo). The normal value of SCCA in our hospital is ≤ 1.5 ng/ml.

Table 1. Background, treatment and prognosis of patients with cervical cancer

Patient	Age	FIGO staging	Treatment	Pathology	Prognosis
CC1	20	Ia1	Conization	SCC	CR
CC2	42	Ia1	Modified radical hysterectomy	SCC	CR
CC3	64	Ia1	Modified radical hysterectomy	SCC	CR
CC4	41	Ia1	Modified radical hysterectomy	SCC	CR
CC5	43	Ia2	Modified radical hysterectomy	SCC	CR
CC6	49	Ib1	Radical hysterectomy	SCC	CR
CC7	83	Ib1	Radiotherapy	SCC	CR
CC8	53	Ib2	Chemoradiotherapy	SCC	CR
CC9	42	Ib2	Radical hysterectomy ^a	SCC	CR
CC10	61	IIa	Chemoradiotherapy	SCC	CR
CC11	65	IIa	Radical hysterectomy ^b	SCC	CR
CC12	54	IIa	Radical hysterectomy ^c	SCC	CR
CC13	62	IIa	Chemoradiotherapy	SCC	CR
CC14	45	IIb	Chemoradiotherapy	SCC	CR
CC15	61	IIb	Not clear ^d	SCC	Not clear
CC16	60	IIb	Chemoradiotherapy	SCC	CR
CC17	56	IIb	Chemoradiotherapy	SCC	CR
CC18	80	IIIb	Radiotherapy	SCC	CR
CC19	65	Iva	Radiotherapy	SCC	PR
CC20	43	IVb	Chemoradiotherapy	SCC	PR

FIGO, The International Federation of Gynecology and Obstetrics; SCC, squamous cell carcinoma; CR, complete response to therapy and no recurrence or metastasis; PR, partial response.

^aPatient received chemoradiotherapy due to invasion of blood vessels after surgery.

^bPatient received chemoradiotherapy after operation.

^cPatient received Neo-adjuvant chemotherapy before operation.

^dTreatment and prognosis are not clear due to changes in hospitals.

ISOLATION OF DNA

Blood samples were centrifuged at 3000 rpm for 5 min at room temperature. The plasma was saved and stored at -20°C until analysis. The DNA was extracted from 200 μl of plasma using a commercial kit (QIAamp DNA Blood Mini Kit, Qiagen, Hilden, Germany) and the DNA was eluted with 50 μl of MilliQ.

QUANTITATIVE REAL-TIME PCR ANALYSIS OF HPV DNA

For analysis of HPV DNA in plasma of patients with cervical cancer, quantitative real-time PCR (qPCR) fluorescent assay for HPV16 was performed using HPV16 primers and