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Developmentally dynamic changes of DNA methylation in the mouse *Snurf/Snrpn* gene

Kazumi Miyazaki [a], Christophe K. Mapendano [b], Tomokazu Fuchigami [c], Shinji Kondo [a], Tohru Ohta [d], Akira Kinoshita [b], Kazuhiro Tsukamoto [c], Ko-ichiro Yoshiura [b], Norio Niikawa [b], Tatsuya Kishino [a,*]

[a] Division of Functional Genomics, Center for Frontier Life Sciences, Nagasaki University, Sakamoto 1-12-4, Nagasaki 852-8523, Japan
[b] Department of Human Genetics, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, 852-8523, Japan
[c] Department of Pharmacotherapeutics, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, 852-8523, Japan
[d] Research Institute of Personalized Health Sciences, Health Sciences University of Hokkaido, Hokkaido, 061-0293, Japan

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ABSTRACT

The mouse *Snurf/Snrpn* gene has two differentially methylated regions (DMRs), the maternally methylated region at the 5′ end (DMR1) and the paternally methylated region at the 3′ end (DMR2). DMR1, a region that includes the *Snrpn* promoter and the entire intron 1, has been thought to be a germline DMR, which inherits the parental-specific methylation profile from the gametes. DMR1 is not only associated with imprinted *Snrpn* expression, but implicated in imprinting control of other genes in the region. We have now characterized the highly conserved activator sequence (CAS) in the *Snrpn* intron 1 among human and rodents and demonstrate that the mouse CAS is not a germline DMR but shows developmentally dynamic changes of DNA methylation and has 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.

1. Introduction

Genomic imprinting is an important mechanism of gene regulation, which causes genetic nonequivalence in expression between maternal and paternal genomes in mammals. Such parent-of-origin specific gene regulation is caused by epigenetic modifications, which initially occur during gametogenesis without any nucleic acids changes (Surani, 1998; Tilghman, 1999). Epigenetic modifications in gametes continue to differentiate alleles of parental origin even after zygote formation, so that one parentally-derived allele eventually becomes preferentially expressed. One of the epigenetic modifications in imprinting is DNA methylation. DNA methylation can be stably inherited in somatic cells and reset in gametes. In the imprinted loci, differentially methylated regions (DMRs) between the maternal and paternal alleles are often found and associated with parent-allele-specific expression. For some imprinted loci, DMRs are gamete-derived methylated regions (germline DMRs), where DNA methylation in the gamete is maintained throughout development in all somatic lineages. However, there exist secondary DMRs, which are acquired during development and associated with primary imprints in the gamete (Constancia et al. 1998). Although the primary DMRs are essential for establishment and maintenance of imprinting, which are associated with the imprinting center (IC) (Bourc'his et al. 2001; Hata et al. 2002), it is unknown whether the secondary DMRs directly control the imprinted expression or exist only as the consequence of an epigenetic event.

The mouse chromosome 7C is a large imprinted domain orthologous to the Prader-Willi syndrome (PWS)/Angelman syndrome (AS) critical region at human chromosome 15q11-q13. The imprinted domain 7C contains paternally expressed genes, *Snurf/Snrpn* (hereafter termed *Snrpn*), *Ndn*, *Magel2*, *Mkrn3* and C/D-box small nucleolar RNAs (snoRNAs), and the maternally expressed gene, *Ube3a* (Nicholls and Knepper, 2001). Imprinted expression within this large domain is coordinated by a bipartite cis-acting IC located upstream from the *Snrpn* gene. In the large imprinted domain, several DMRs have been identified. One of them is in the *Snrpn* locus, which has two DMRs (Fig. 1A), the 5′ end methylated on the maternal allele (DMR1) and the 3′ end methylated on the paternal allele (DMR2) (Shemer et al. 1997; Gabriel et al. 1998). DMR1 is a ~6 kb region containing the 5′ end of the *Snrpn* gene and the entire *Snrpn* intron 1 (Fig. 1A). DMR2 is a 3.5-kb region spanning exons 7–10. Both DMR1 and DMR2 are thought as germline DMRs, which inherit the parental-specific methylation profile from the

Abbreviations: AS, Angelman syndrome; CAS, conserved activator sequence; ChIP, Chromatin immunoprecipitation; DHS, nuclease hypersensitive sites; DMR, differentially methylated region; IC, imprinting center; PWS, Prader-Willi syndrome; SMP, *Snrpn* minimal promoter; UPD, uniparental disomy.

* Corresponding author. Fax: +81 95 819 7178.
E-mail address: kishino@nagasaki-u.ac.jp (T. Kishino).

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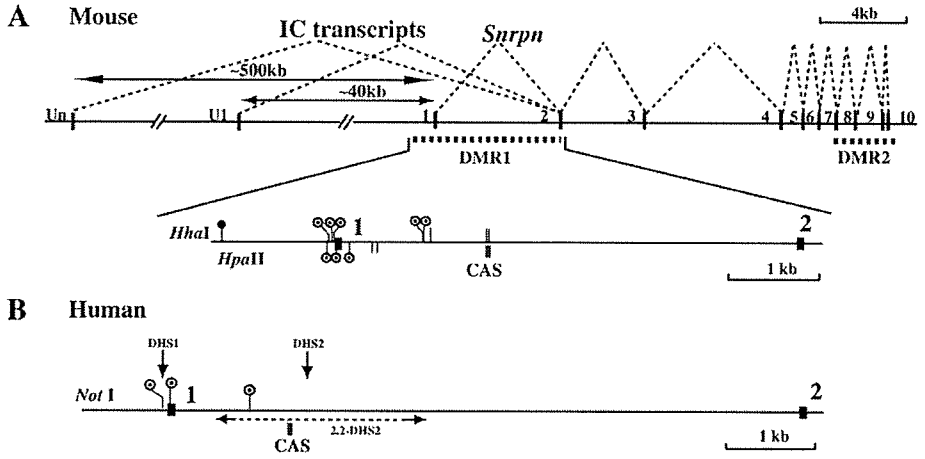

Fig. 1. Genomic organization and the DNA methylation pattern of the mouse (A) and human (B) *Snrpn/SNRPN* locus. Black and gray boxes indicate exons and the CAS region. (A) IC transcripts overlap with *Snrpn* except exon 1. Spliced exons except U exons in IC transcripts are omitted. Short vertical lines 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

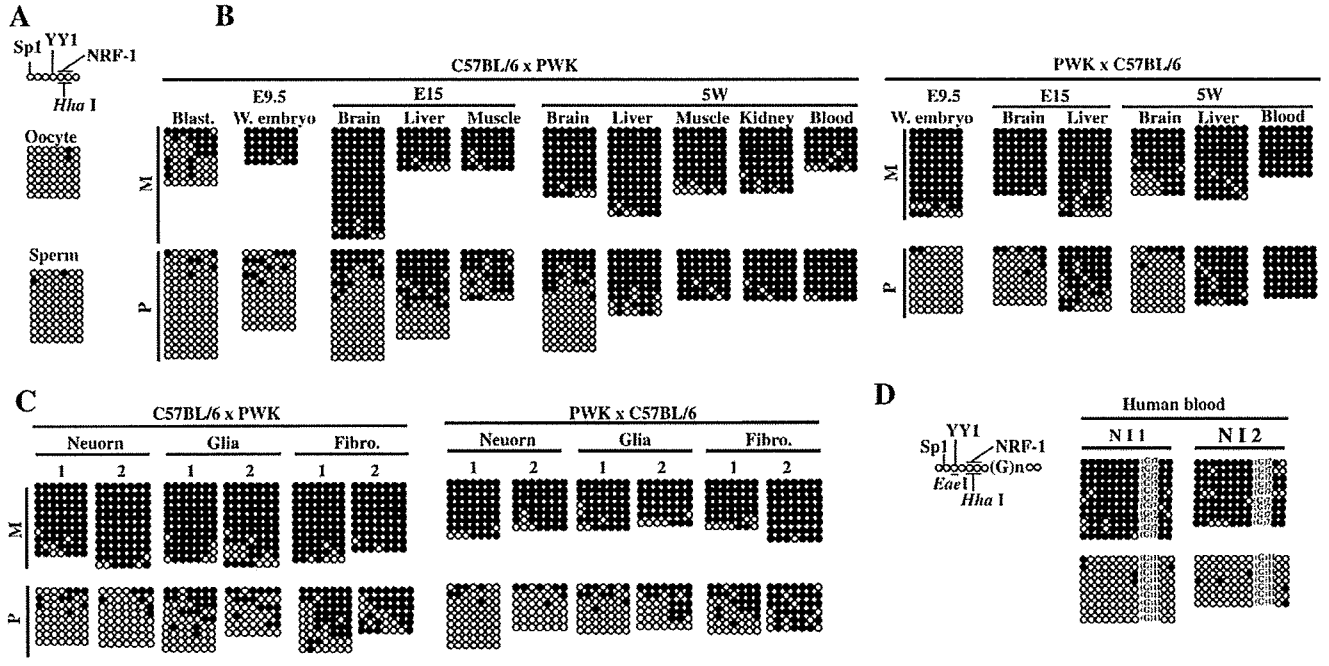

Fig. 2. Methylation of CpG dinucleotides in the CAS in gametes, tissues and culture cells in mice (A, B, C) and in human lymphocytes (D). Each row of dots represents the series of CpGs in an individual sequence molecule, in which methylated CpGs are shown as solid circles and unmethylated CpG as open 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 *Hhal* 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 *Hhal* 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 *Hhal* 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 *SssI* methylase resulted in a complete

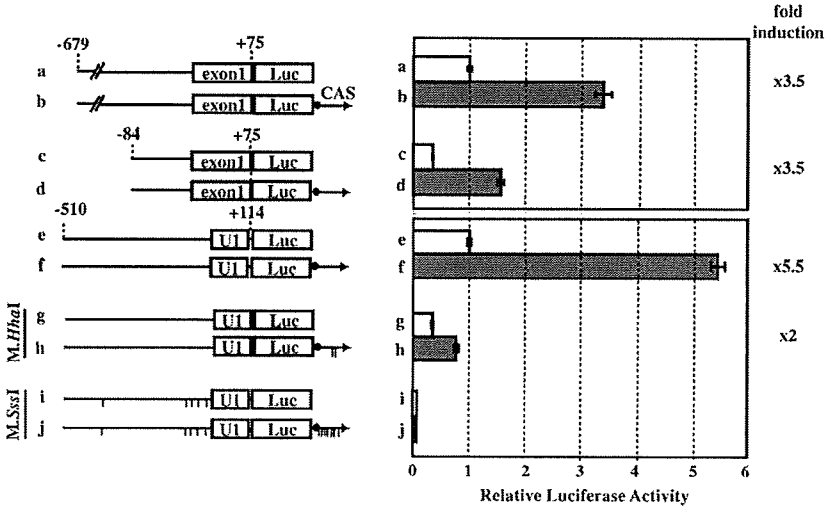

Fig. 3. Methylation-sensitive enhancer activity of the mouse CAS. Reporter constructs with the *Snrpn* promoter (a, b), the *Snrpn* minimal promoter (c, d), and the U1 promoter (e, f, g, h, i, j) lacking or carrying the mouse CAS (arrow) were assayed by transient expression assays. In the constructs (g, h, i, j), *in vitro* methylation with *Hhal* (g, h) or *SssI* (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 *Hhal* 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 *Hhal* 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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RESEARCH ARTICLE

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A Locus for Ophthalmo-Acromelic Syndrome Mapped to 10p11.23

**Haruka Hamanoue,[1,2] Andre Megarbane,[3] Takaya Tohma,[4] Akira Nishimura,[1] Takeshi Mizuguchi,[1] Hirotomo Saitsu,[1] Haruya Sakai,[1] Shoko Miura,[5] Tatsushi Toda,[6] Noriko Miyake,[1] Norio Niikawa,[5] Koichiro Yoshiura,[5] Fumiki Hirahara,[2] and Naomichi Matsumoto[1]*

[1]Department of Human Genetics, Yokohama City University Graduate School of Medicine, Yokohama, Japan

[2]Department of Obstetrics and Gynecology, Yokohama City University Graduate School of Medicine, Yokohama, Japan

[3]Medical Genetics Unit, St. Joseph University, Beirut, Lebanon

[4]Division of Pediatrics, Okinawa Prefectural Nanbu Medical Center & Children's Medical Center, Haebaru, Japan

[5]Department of Human Genetics, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

[6]Division of Clinical Genetics, Department of Medical Genetics, Osaka University Graduate School of Medicine, Suita, Japan

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Ophthalmo-acromelic syndrome (OAS, OMIM %206920) is a rare autosomal recessive disease, presenting with clinical anophthalmia and limb anomalies. We recruited three OAS families including a Japanese family with two affected patients and two consanguineous Lebanese families each having an affected. Homozygosity mapping was performed using the 50K SNP chip and additional informative markers. A locus for OAS was mapped to the 422-kb region at 10q11.23, based on the results from the two consanguineous families as well as the consistent data from the Japanese non-consanguineous family. The 422-kb region only contained one gene, *MPP7*. Although we could not detect any pathological mutations in OAS families analyzed, *MPP7* could remain a candidate as aberrant changes might exist beyond our mutation detection methods. Further families are needed to confirm this candidate locus. © 2009 Wiley-Liss, Inc.

Key words: ophthalmo-acromelic syndrome; linkage study; genetic locus; 10p11.23

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INTRODUCTION

Ophthalmo-acromelic syndrome (OAS, OMIM %206920), also known as Waardenburg's recessive anophthalmia syndrome, is a rare autosomal recessive disorder, presenting with anophthalmia or microphthalmia and limb anomalies. Since the first report by Waardenburg [1961], at least 35 cases from 21 families have been reported [Richieri-Costa et al., 1983; Pallotta and Dallapiccola, 1984; Traboulsi et al., 1984; Le Merrer et al., 1988; al Gazali et al., 1994; Quarrell, 1995; Sayli et al., 1995; Suyugul et al., 1996; Megarbane et al., 1998; Cogulu et al., 2000; Tekin et al., 2000; Caksen et al., 2002; Kara et al., 2002; Garavelli et al., 2006; Teiber et al., 2007]. Majority of OAS families are consanguineous (~90%) [Garavelli et al., 2006]. Ocular phenotypes in OAS widely range from mild microphthalmia to true anophthalmia [Kara et al., 2002]. Typical limb malformations include fused 4th and 5th metacarpals and short 5th finger in hands and olygodactyly in foot (four toes) [Teiber et al., 2007]. Most cases have bilateral anophthalmia/microphthalmia (88%), but unilateral abnormality is also noted. Other (visceral) malformations are rare, but venous or vertebral anomaly was recognized each in single cases [Tekin et al., 2000; Teiber et al., 2007].

The genetic cause for OAS remains undetermined. Genome-wide homozygosity mapping was undertaken and a locus for OAS was identified using three families including four affected individuals. A possible responsible gene will be discussed.

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*Correspondence to:
Naomichi Matsumoto, M.D., Ph.D, Department of Human Genetics, Yokohama City University Graduate School of Medicine, Fukuura 3-9, Kanazawa-ku, Yokohama 236-0004, Japan.
E-mail: naomat@yokohama-cu.ac.jp

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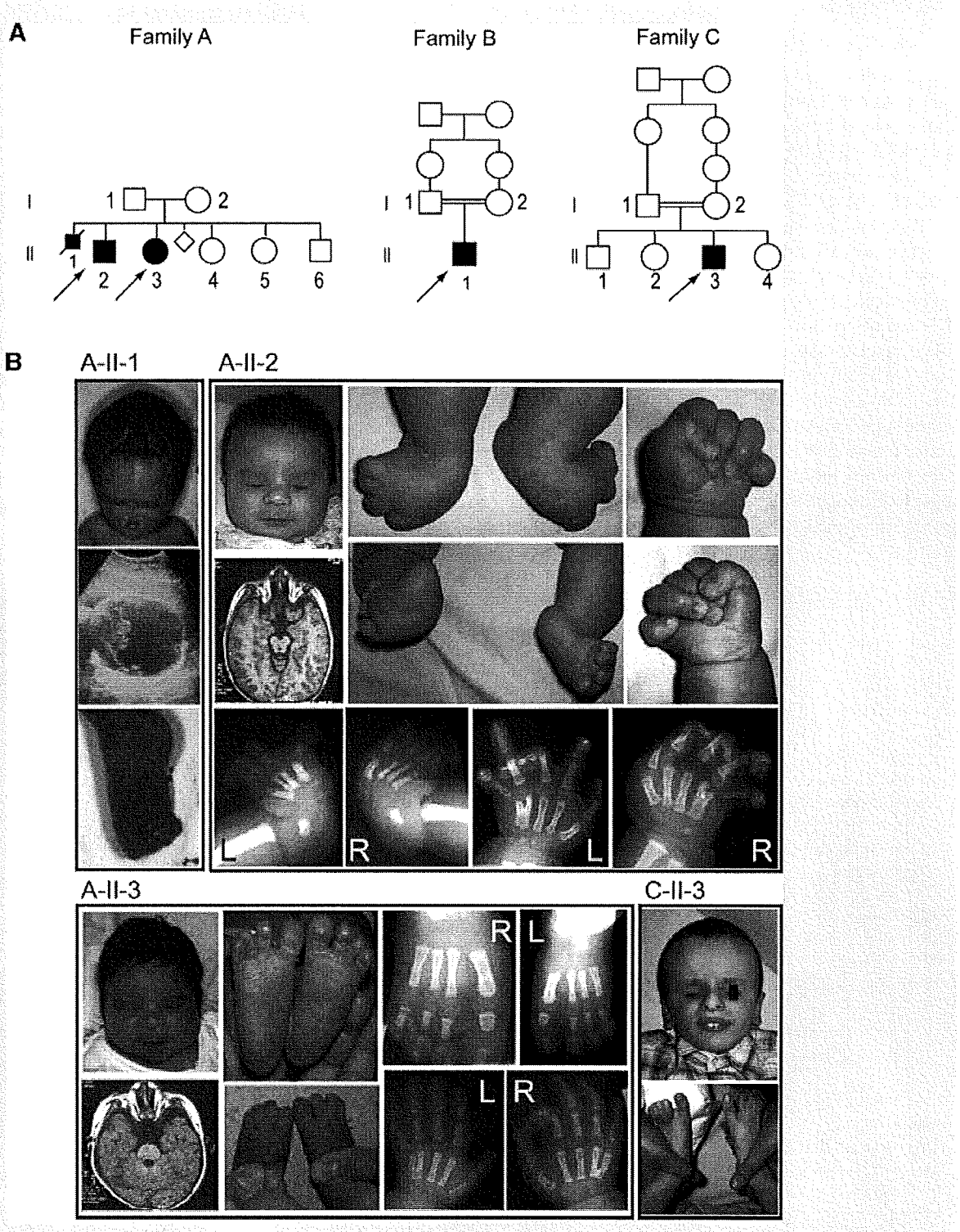

FIG. 1. Three OAS families used for this study and clinical manifestations of families A and C. A: Three OAS-family pedigrees are shown. Family A (Japanese) has two affected individuals and one affected abortus, family B (Lebanese) one affected born from the consanguineous parents, and family C (Lebanese) one affected also from the consanguineous parents. B: Clinical features of one affected abortus (A-II-1) and two affected individuals (A-II-2 and A-II-3) in family A and an affected individual (C-II-3) in family C. A-II-1 showed bilateral microphthalmia, adherent eyelids, bilateral oligodactyly (absence of 1st toes), cleft lip/palate and holoprosencephaly with hydrocephaly. A-II-2 at age of 6 years and A-II-3 at 5 years both presented with bilateral anophthalmia (confirmed by brain MRI), closed eyelids and limb anomalies with bilateral metacarpal synostosis between the 4th and 5th fingers and bilateral oligodactyly (absence of 1st toes). C-II-3 at age of 2 years showed bilateral anophthalmia and distal limb abnormalities including bilateral syndactyly of 2nd to 5th toes with symphysis.

MATERIALS AND METHODS

Patients and Their Families

Three families with one or two cases of OAS were analyzed in this study (Fig. 1 and Table I). In family A, two affected sibs (A-II-2 at 6 years and A-II-3 at 5 years) both presented with bilateral anophthalmia, closed eyelids and limb anomalies with bilateral metacarpal synostosis between the 4th and 5th fingers and bilateral oligodactyly (absence of 1st toes). In this family, their first child was a stillborn son (A-II-1), who had bilateral microphthalmia, adherent eyelids, bilateral oligodactyly (absence of 1st toes), cleft lip/palate and holoprosencephaly with hydrocephaly. Though no evidence of consanguinity was obtained, their eight grandparents were all originated from the Okinawa island in Japan. The other two families (B and C) were independent consanguineous Lebanese families, of which the family B was previously reported [Megarbane et al., 1998]. The proband B-II-1, a 10-year-old boy, was the first child of healthy parents, presenting with bilateral anophthalmia, adherent eyelids, deeply set orbits, anteverted nares, normally positioned ears, a normal philtrum, thin lips, micrognathia, very short neck, narrow chest, bilateral upper limb anomalies (split hands: four digits on the right hand and two digits on the left), and six short toes on the right foot (right postaxial polydactyly) [Megarbane et al., 1998]. These limb abnormalities are atypical for OAS. The patient, C-II-3, a 2-year-old boy, showed bilateral anophthalmia and distal limb abnormalities including bilateral syndactyly of 2nd to 5th toes. A total of 4 affected and 12 unaffected members from the three families were analyzed. Genomic DNA was obtained from peripheral blood leukocytes using Quick-Gene 610-L (FUJIFILM, Tokyo, Japan) after informed consent. Experimental protocols were approved by the Institutional Review Board at Yokohama City University School of Medicine.

SNP Genotyping

Whole SNP genotyping was undertaken using the GeneChip™ Human Mapping 50K Array *Xbal* (Affymetrix, Inc., Santa Clara, CA) containing 58,625 SNPs (single nucleotide polymorphism) according to the manufacture's protocols. In brief, 250 ng DNA was digested with *Xba*I. The adaptors were ligated to the digested DNA, and the ligation-mediated PCR with single-primer was performed. PCR products were purified by microcon YM-100 (Millipore, Inc., Billerica, MA). The product was fragmented, end-labeled, and hybridized to an array. SNP markers on the chip are almost equally distributed in the whole genome, with mean marker-distances of

TABLE I. Clinical Features of OAS Patients

	A-II-1	A-II-2	A-II-3	B-II-1	C-II-3
Origin	Okinawa, Japan	Okinawa, Japan	Okinawa, Japan	Lebanon	Lebanon
Consanguinity	−	−	−	+	+
Sex	Male	Male	Female	Male	Male
Eye abnormality	+	+	+	+	+
Anophthalmia	Bilateral	Bilateral	Bilateral	Bilateral	Bilateral
Loss of optic nerve (CT)	n.c.	Bilateral	Bilateral	Bilateral	Bilateral
Loss of optic tract (CT)	n.c.	+	+	−	−
Upper limb abnormality	+	+	+	+	+
Oligodactyly	n.c.	−	−	Right oligodactyly/ left lobster-claw	−
Metacarpal synostosis	n.c.	4th and 5th fingers	4th and 5th fingers	2nd and 3rd fingers	−
Clinodactyly	+	+	−	+	+
Camptodactyly	n.c.	+	−	+	+
Single transverse palmar crease	n.c.	+	+	n.c.	−
Lower limb abnormality	+	+	+	+	+
Oligodactyly/polydactyly/syndactyly	Bilateral oligodactyly	Bilateral oligodactyly	Bilateral oligodactyly	Right polydactyly	Bilateral syndactyly
Metatarsal synostosis	n.c.	+	+	+	−
Tibia valga	n.c.	+	+ (mild)	n.c.	−
Hypoplastic fibula	n.c.	+	+ (mild)	n.c.	−
Abnormal cleavage between toes	n.c.	1st and 2nd toes	1st and 2nd toes	−	1st and 2nd toes
Dermal syndactyly	n.c.	2nd and 3rd toes	2nd and 3rd toes	−	2nd to 5th toes
Talipes valgus	−	+	−	+	−
Other					
Holoprosencephaly	+	−	−	−	−
Cleft palate	+	−	−	−	−
Failure to thrive	n.c.	+	+	+	+
Developmental retardation	n.c.	DQ = 10	DQ = 15	+	+
Cryptorchidism	n.c.	Right		−	n.c.

n.c., not confirmed.

23.6 kb and an average heterozygosity of 0.30 among African American, European, and Asian. SNP calling, signal intensity data, and Mendelian error in each pedigrees to exclude conflicted SNPs were checked using GCOS 1.2 (the GeneChip Operating Software) platform (Affymetrix) and the Batch analysis in GTYPE 4.0 (GeneChip Genotyping Analysis Software) (Affymetrix), with the default setting for mapping algorithm. CNAG (Copy Number Analyser for GeneChip) Ver. 2.0 was also used to validate copy number alterations as well as loss of heterozygosity (LOH).

Linkage Analysis

Multipoint linkage analysis using aligned SNPs was performed using ALLEGRO software [Gudbjartsson et al., 2000, 2005]. Two-point linkage analysis of candidate regions was also performed using the LINKAGE package, MLINK (FASTLINK software, ver. 5.1). In each program, autosomal recessive model of inheritance with complete penetrance and a disease allele frequency of 0.001 were applied.

Fine Mapping With Short Tandem Repeat Markers

Fine mapping of possible candidate regions using additional microsatellite markers was done as previously described [Kondo et al., 2004]. Most markers were designed according to the Marshfield genetic map (http://research.marshfieldclinic.org/genetics). If appropriate markers were not found, candidate di-, tri-, and tetranucleotide repeat markers were originally selected from regions of interest using the UCSC genome browser (March 2006 assembly), and PCR primers were designed with the Primer3 program.

TABLE II. Primer Information of Markers

Marker	Forward (5′ > 3′)	Reverse (5′ > 3′)	Fluorescence	Product size (bp)	Annealing temperature (°C)
D10S1653[a]	CCTTTGGATAAAGCCTCCT	TATCATTGTCTCATCCGGG	VIC	201–213	55
D10S1661[a]	ACGCTACTTGCCAGGTC	ATTGCTTCCCTGAGAGTGT	VIC	250–272	58
D10S1476[a]	TGACTAAACAGACCCAGACTTG	GAACGCATGTCCACCCTA	NED	250–266	62
D10S504[a]	TCAGGTATTTCTTCATAGCAG	TTCCCTTGCTCTGCAGCTT	NED	366–370	62
D10S1125[a]	TGGTGGCCTCTTACCTAG	CCATTTGTATGTGTTCTCTTGAG	NED	225–249	64
D10S466[a]	CTGGGCCACAGTGAGACT	TAGGTCATCTGGTCTTCCATAC	NED	120–140	60
D10S1734[a]	GCCTGGGTGACAGAGTGAGATTCTA	ACACACGTACACATGGGGTGGT	NED	163–189	64
D10S1789[a]	TTTCCCACTCCAGTGC	TCATAGATAGAGACCATTAGTTTCA	VIC	134–150	60
D10S1673[a]	CCAACCTGGATGACAGAGC	CTTACCCCCAACCAAAGGAC	VIC	198–216	64
D10S1747[a]	TGTAGACCAGTAGACCATGACAAAT	CCACAGTCAGATATAGTGTGCAA	FAM	117–123	64
STS1[c]	TGTCCATCTCTTCAAACACTGG	TCCTGGGAATAGCGTTCACT	NED	243	60
AFM290XE1[a]	ATTTTTGACATTGTCCCA	CTAAGCCCTAGCACCTTT	FAM	247	57
AFM295TH1[a]	TATTATACTCCAGCCGGGG	GGAGACTATTTACTTTGTGTCCTTG	FAM	130	63
STS2[c]	TGGATATGAAAAGGGGTGATAA	GGTCCAGATGGTACTCACACG	FAM	246	60
STS3[c]	TGGGCTGCACATTTATACCA	TGTGACCTGCTCCTCACAAG	FAM	169	60
STS4[c]	TGATGTGTGTGTATTTTGTGTGTG	ACTCTTTGCAGCATCCCAGT	FAM	226	60
STS5[c]	TCTGTGTGCAGCTCCTCAGT	ACCTGGACAGGATCATCTGG	NED	224	60
D10S572[a]	CAGTGATTTTAGACAGGGATTTTA	AATTATGATACTTATTGATGGGGA	NED	275–283	63
D10S2481[b]	TGGAAGTTATGGACCAGGAA	CCAATGTCCAGCTAAGTGAGG	VIC	312	60
D10S197[a]	ACCACTGCACTTCAGGTGAC	GTGATACTGTCCTCAGGTCTCC	FAM	161–173	55
STS6[c]	TGCATTTAAGGAGAATCAGTTG	GCAGTGACTGTTCAAGATTTTTGT	FAM	194	60
STS7[c]	TGAACTACTGCTCTCAAATCTGTGT	GGGACACAATGGCTTTGAAC	VIC	157	60
STS8[c]	TTGGATTTTATTTGAAAATTAGGG	TTGGTTGGCTGAATAACTTCC	FAM	213	60
STS9[c]	GCTAATCCAGAGATACCACCAGA	CCTAGTTTGCTGAGACTGTTGTG	NED	371	60
STS10[c]	CCCTAGAAGTATTTGAAGAAGTAGCA	TGTGGTGCTCTTTCCTGTGA	NED	108	60
STS11[c]	GCTCAGTGGGACAATTCATGT	GAATAATGCCCCCGAAAGAT	VIC	399	60
STS12[c]	GCTGTTGGCTGTGAGTTCAA	CCCTTGGGCTTGGACTAGAA	FAM	388	60
UT541[a]	ATGGGGGTAGAGGGTCTGG	CAGCCTGGGTGACAAAGTCT	NED	143–340	60
AFMB345YA9[a]	GGAACCTAAGGCATGTTGAT	CCAAGACCCTGTCTGAAAAA	FAM	151–171	60
GATA29G05[a]	TGCTTATATCCAGCTAATATAAATG	CCATGAGGTTTATTTTCCCC	FAM	108–172	60
AFM095ZH7[a]	TATATGCAGTTTGGGATGGG	ATTGGGCTGTGCCTACACTT	VIC	213–231	60
D10S208[a]	AGGTGACTGTTTTGGGGGAG	GAGTGTGGGGATGTTTTCAA	NED	170–186	55
AFM137XH4[a]	AACATCCATTTGGAGAATTAAAAT	TACAGTGTGATTGCACGACT	NED	171–183	60
AFM353TB5[a]	TCAGTGGGAACGTAAATCAG	AGCTGAATATTATTCCATTGTGAGT	NED	175	58
D10S196[a]	TTCAAAGGTGGAGACCCTTC	TTTTGGTCAGAATGGAGTGG	VIC	99–109	55

[a]Primers for known microsatellite marker.
[b]Primers for known microsatellite marker with our modification.
[c]Newly designed primers.

Fluorescent-labeled (either FAM, VIC, or NED) forward primers and tailed reverse primers were purchased from Applied Biosystems (Tokyo, Japan). PCR products for each marker were electrophoresed on ABI Prism Genetic Analyzer 3100 (Applied Biosystems), and analyzed using GeneMapper Software ver. 3.5 (Applied Biosystems). PCR was cycled 40 times at 94°C for 30 sec, at 55–64°C for 30 sec and at 72°C for 30 sec in a total volume of 10 μl, containing 30 ng of genomic DNA as a template, 0.5 μM of each primer, 200 μM of each dNTP, 1× ExTaq buffer and 0.25 U ExTaq (Takara Bio, Inc., Ohtsu, Japan). Haplotype blocks were constructed manually. The list of primers is presented in Table II.

Mutation Analysis of a Candidate Gene

All coding exons and exon-intron boundaries together with 5′- and 3′-untranslated regions of *MPP7* (Membrane protein, palmitoylated 7) were analyzed. PCR was cycled 35 times at 94°C for 30 sec, at 60°C for 30 sec and at 72°C for 30–90 sec in a total volume of 20 μl containing 30 ng of genomic DNA as a template, 0.5 μM of forward and reverse primers, 200 μM of each dNTP, 1× ExTaq buffer, and 0.25 U of ExTaq (Takara Bio, Inc.). All primers were designed using the Primer3 software. Detailed information of *MPP7* primers is available on request. PCR products were purified with ExoSAP™ (USB Co., Cleveland, OH) and sequenced using BigDye Terminator 3.1 (Applied Biosystems) on the 3100 Genetic Analyzer. Sequences of patients were compared to the reference genome sequences in the UCSC Genome Browser (Mar 2006 assembly) using the Seqscape software ver. 2.1 (Applied Biosystems).

Expression Study of Mpp7 in Mouse Tissues

Whole head tissues from mice embryos at E10.5, eyeball, forelimb and hindlimb tissues from embryos each at E12.5, 13.5, and 16.5 were collected. Total RNA was extracted from collected tissues using Trizol reagent (Invitrogen, Carlsbad, CA). Two micrograms of RNA was reverse-transcribed in a volume of 20 μl using PrimeScript 1st strand cDNA Synthesis kit (Takara Bio, Inc). RT-PCR analysis was started at 94°C for 5 min as a first denaturing step, then cycled 25, 30, or 35 times at 94°C for 30 sec, at 56°C for 30 sec and at 72°C for 30 sec in a volume of 25 μl, containing 2 μl of reverse transcription reaction as a template, 0.2 μM of each primer, 400 μM of each dNTP, 1× ExTaq buffer and 0.125 U ExTaqHS (Takara Bio, Inc). Primers for RT-PCR were as follows: *Mpp7*-1-F, 5′-TGTATGAGCTGTTGGCTGCT-3′, and *Mpp7*-1-R, 5′-AGCCTT-

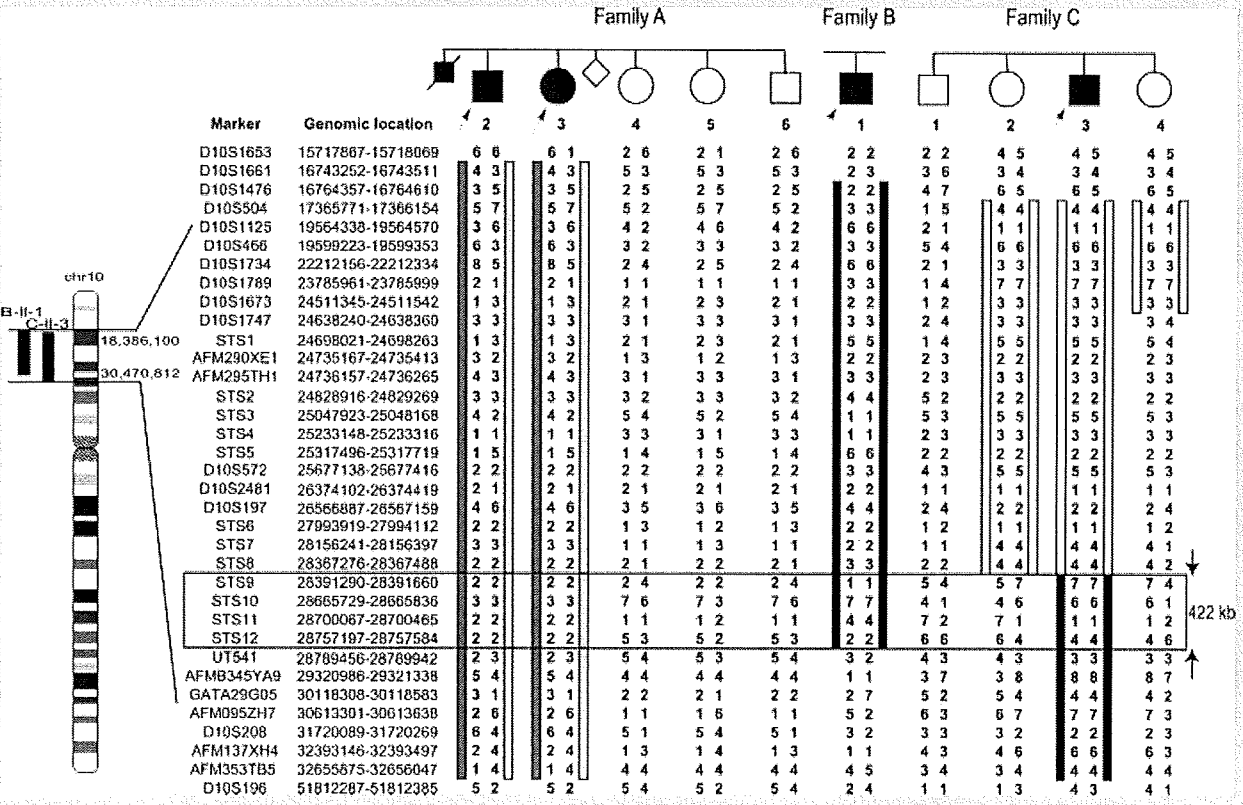

FIG. 2. Homozygosity mapping and haplotypes of three OAS families. Pedigrees of the three OAS families (Top). Chromosome 10 ideogram and IBD regions of B-II-1 and C-II-3 revealed by Affymetrix GeneChip 50K array are indicated with black bars. Polymorphic markers around IBD regions and haplotypes are indicated in all families. In families B and C, the common IBD region was mapped to a 422-Kb region, flanked by marker *STS8* and *UT541*. Affected sibs in Family A (II-2 and II-3) possess heterozygous haplotype blocks (shown as gray bars), probably implying compound heterozygous mutations.

GACATTGGGTTTTG-3′; *Mpp7*-2-F, 5′-AGCCTTGACATTGGG-TTTTG-3′, and *Mpp7*-2-R, 5′-TTATGAATGTAACCAACTCAC-TGG-3′. To ensure equal loading of cDNA into RT-PCR reactions, *B2m* cDNA was amplified as an internal control using the following primers: *B2m*-F, 5′-TGGTGCTTGTCTCACTGACC-3′, and *B2m*-R, 5′-TGCTTAACTCTGCAGGCGTAT-3′.

RESULTS AND DISCUSSION

Linkage analysis using ALLEGRO program revealed 16 candidate loci showing the LOD score ($\theta = 0.000$) higher than 3.0 (data not shown). All candidate regions except for a region of chromosome 10 (SNP blocks from rs7920803 to rs7094225 with the maximum LOD score, 3.9880) were ruled out by additional microsatellite markers as they did not show a pattern of identical by descent (IBD). LOH analysis using the CNAG program confirmed that B-II-1 and C-II-3 shared only one consistent region at chromosome 10. The region was comprised of 303 consecutive SNPs from rs1986480 to rs10508745, spanning approximately an 11-Mb segment at 10p12.33–p11.23 (Fig. 2). In contrast, A-II-2 and A-II-3 did not show any blocks of IBD, suggesting that it is unlikely that that their parents had a common ancestor. Further fine mapping using more markers confirmed that an affected individual (C-II-3) and an unaffected individual (C-II-2) shared the same homozygous genotype's region from *D10S504* to *STS8*. Thus the candidate region was narrowed down to a 422-kb segment from *STS9* and *STS12* at 10p11.23 through the analysis of families B and C. ALLEGRO program using all families indicated the maximum multi-point LOD score was 3.9863 near *STS9*. MLINK showed a maximum two-point LOD score was 2.9444 ($\theta = 0.000$) at *STS10*. Within the 422-kb region, only one established gene, *MPP7* (palmitoylated membrane protein 7) was located. Mutation screening of *MPP7* could not detect any abnormalities. All the haplotypes of the 422-kb segment in the three families are different.

Homozygosity mapping of two consanguineous Lebanese families each having an affected child revealed a candidate locus from *STS9* to *STS12*, a 422-kb region under the hypothesis that OAS is an autosomal recessive disorder. Though we initially expected that broad and many chromosomal regions might be highlighted as IBD, only a narrow 10p11.23 segment turned out to be a region of interest. Two affected sibs (A-II-1 and A-II-2) were likely to have less and smaller IBD regions as the family was not consanguineous. Indeed, we could not confirm any IBD regions on the Affymetrix 50K SNPs, suggesting that the two patients in the family A have compound heterozygous mutations.

Although *MPP7* is the only gene mapped to the 440-kb region and *Mpp7* is expressed in head tissues at E10.5, and in eyeballs and limbs at E12.5–E16.5 (Fig. 3), no causative mutations were found in our patients. This could be due to the limit of analytical methods. For example, mutations of promoter regions or small intragenic deletions/duplications might not be detected, though the Affymetrix GeneChip 50K could not detect any copy number changes of the region. Further investigation is absolutely necessary.

OAS is a very rare syndrome, usually the eye sign is consistent but the limb abnormalities are variable, so it is possible that OAS may have locus heterogeneity with different subtypes. As families B and

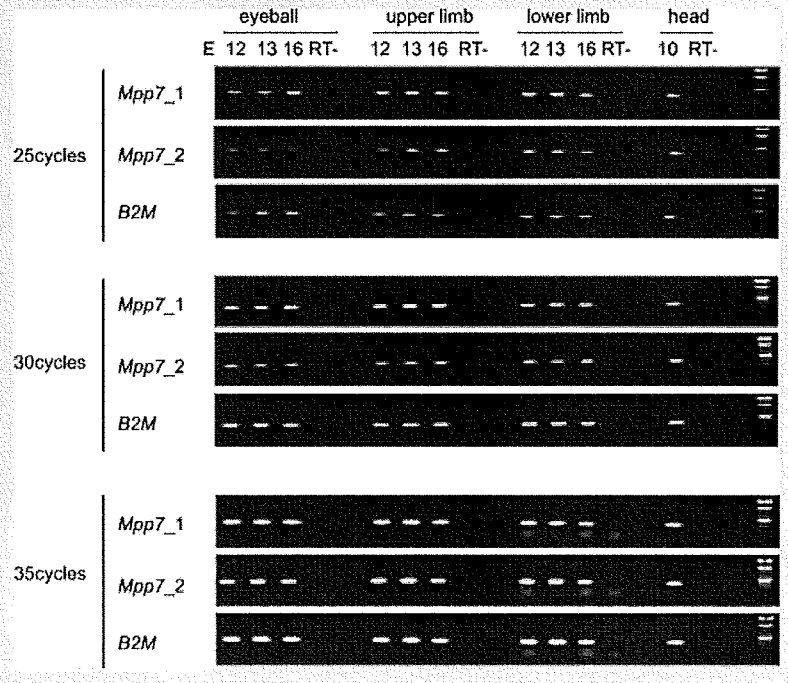

FIG. 3. Mpp7 expression analysis in mice embryonic tissues. *Mpp7* expression is confirmed using two different primer sets in all tissues examined by 25-, 30-, 35-cycled RT-PCR. Eyeball, upper and lower limb tissues at E12.5, E13.5, and E16.5, and whole head tissues at E10.5 were examined. *B2M* is an internal control gene.

C are of the same ethnic origin and were clinically examined by one of coauthors (M.A), we expected the similar haplotype for the two families. However, haplotypes of the 422-kb homozygous region are different between the two families. Thus the two families may not be caused by the same ancestral mutation. More families, if available, should be investigated.

In conclusion, a locus for OAS is validated by SNP homozygosity mapping. It is a 422-kb segment at 10p11.23 corresponding to *MPP7*, but no mutation was found. The SNP homozygosity mapping is very useful for autosomal recessive traits even if only a few families are available for study, but of course, more patients and families are definitely useful for this type of analysis.

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ORIGINAL ARTICLE

Molecular karyotyping in 17 patients and mutation screening in 41 patients with Kabuki syndrome

Hideo Kuniba[1,2,14], Koh-ichiro Yoshiura[1,14], Tatsuro Kondoh[2], Hirofumi Ohashi[3,14], Kenji Kurosawa[4], Hidefumi Tonoki[5], Toshiro Nagai[6,14], Nobuhiko Okamoto[7], Mitsuhiro Kato[8], Yoshimitsu Fukushima[9,14], Tadashi Kaname[10,14], Kenji Naritomi[10,14], Tadashi Matsumoto[2], Hiroyuki Moriuchi[2], Tatsuya Kishino[11,14], Akira Kinoshita[1,14], Noriko Miyake[12,14], Naomichi Matsumoto[12,14] and Norio Niikawa[1,13,14]

The Kabuki syndrome (KS, OMIM 147920), also known as the Niikawa–Kuroki syndrome, is a multiple congenital anomaly/mental retardation syndrome characterized by a distinct facial appearance. The cause of KS has been unidentified, even by whole-genome scan with array comparative genomic hybridization (CGH). In recent years, high-resolution oligonucleotide array technologies have enabled us to detect fine copy number alterations. In 17 patients with KS, molecular karyotyping was carried out with GeneChip 250K NspI array (Affymetrix) and Copy Number Analyser for GeneChip (CNAG). It showed seven copy number alterations, three deleted regions and four duplicated regions among the patients, with the exception of registered copy number variants (CNVs). Among the seven loci, only the region of 9q21.11-q21.12 (~1.27 Mb) involved coding genes, namely, transient receptor potential cation channel, subfamily M, member 3 (*TRPM3*), Kruppel-like factor 9 (*KLF9*), structural maintenance of chromosomes protein 5 (*SMC5*) and MAM domain containing 2 (*MAMDC2*). Mutation screening for the genes detected 10 base substitutions consisting of seven single-nucleotide polymorphisms (SNPs) and three silent mutations in 41 patients with KS. Our study could not show the causative genes for KS, but the locus of 9q21.11-q21.12, in association with a cleft palate, may contribute to the manifestation of KS in the patient. As various platforms on oligonucleotide arrays have been developed, higher resolution platforms will need to be applied to search tiny genomic rearrangements in patients with KS.
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Keywords: Kabuki syndrome; microdeletion; molecular karyotyping; mutation screening; Niikawa–Kuroki syndrome

INTRODUCTION

Kabuki syndrome (KS, OMIM 147920), also known as Niikawa–Kuroki syndrome, is a multiple congenital anomaly/mental retardation (MCA/MR) syndrome characterized by a distinct facial appearance, skeletal abnormalities, joint hypermobility, dermatoglyphic abnormalities, postnatal growth retardation, recurrent otitis media and occasional visceral anomalies.[1,2] The prevalence was estimated to be 1/32 000 in Japan[3] and 1/86 000 in Australia and New Zealand.[4] Although most cases were sporadic, at least 14 familial cases have been reported. It is assumed that KS is an autosomal dominant disorder, considering the equal male-to-female ratio of patients and parent–child transmission pattern in some familial cases.[5]

The cause of KS remains unknown, even though at least 400 patients have been diagnosed in a variety of ethnic groups since 1981.[3–7] Some works have ruled out several loci; for example, 1q32–q41, 8p22–p23.1 and 22q11, as candidates for KS.[8–13] A study of array-based comparative genomic hybridization (CGH) showed a disruption of the *C20orf133(MACROD2)* gene by ~250 kb deletion in a patient with KS,[14] but the following mutation screening for the gene failed to find a pathogenic base change within exons in 19 other patients with KS[14] and in 43 Japanese patients.[15] Another study of array CGH with 0.5–1.2 Mb resolution reported that 2q37 deletions were detected in two patients with Kabuki-like features, but their facial features were not typical for KS.[16] To date, no concordant specific lesion has been

[1]Department of Human Genetics, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan; [2]Department of Pediatrics, Nagasaki University School of Medicine, Nagasaki, Japan; [3]Division of Medical Genetics, Saitama Children's Medical Center, Iwatsuki, Japan; [4]Division of Medical Genetics, Kanagawa Children's Medical Center, Yokohama, Japan; [5]Department of Pediatrics, Tenshi Hospital, Sapporo, Japan; [6]Department of Pediatrics, Dokkyo University School of Medicine Koshigaya Hospital, Koshigaya, Japan; [7]Department of Planning and Research, Osaka Medical Center and Research Institute for Maternal and Child health, Osaka, Japan; [8]Department of Pediatrics, Yamagata University School of Medicine, Yamagata, Japan; [9]Department of Medical Genetics, Shinshu University School of Medicine, Matsumoto, Japan; [10]Department of Medical Genetics, University of the Ryukyus, Nishihara, Japan; [11]Division of Functional Genomics, Center for Frontier Life Sciences, Nagasaki University, Nagasaki, Japan; [12]Department of Human Genetics, Yokohama City University Graduate School of Medicine, Yokohama, Japan; [13]Research Institute of Personalized Health Sciences, Health Sciences University of Hokkaido, Tobetsu, Japan and [14]Solution Oriented Research for Science and Technology (SORST), Japan Science and Technology Agency (JST), Tokyo, Japan
Correspondence: Dr K-i Yoshiura, Department of Human Genetics, Nagasaki University Graduate School of Biomedical Sciences, Sakamoto 1-12-4, Nagasaki 852-8523, Japan.
E-mail: kyoshi@nagasaki-u.ac.jp
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found by whole-genome scan with array CGH in a bacterial artificial chromosome (BAC) clone with 0.5–1.5 Mb resolution.[16–18]

Chromosomal aberration analysis by high-resolution oligonucleotide array technologies in recent years, called molecular karyotyping, enables us to detect submicroscopic pathogenic copy number alterations, which were undetectable even by BAC array CGH.[19,20] As not a few MCA/MR syndromes are because of chromosomal copy number aberration, we hypothesize that some sort of microdeletion/microduplication causes KS. Herein, we report the results of molecular karyotyping in 17 patients using GeneChip 250K array and those of mutation screening of candidate genes in 41 patients with KS in Japan.

MATERIALS AND METHODS

Subjects

The subjects for molecular karyotyping consisted of 18 patients (nine girls and nine boys) at entry. The subjects for mutation screening consisted of 41 patients (20 girls and 21 boys), including the aforementioned 18 patients. The diagnoses of KS were confirmed by experts of clinical genetics, although written permission for the use of facial photographs in publications was not obtained. These Japanese patients showed a normal karyotype at a 400-band level, and were earlier reported with no pathogenic genome copy number change by 1.5 Mb-resolution BAC array CGH.[18] Genomic DNA was isolated by the standard method from their peripheral blood leukocytes or in part from their lymphoblastoid cell lines. Experimental procedures were approved by the Committee for the Ethical Issues on Human Genome and Gene Analysis at Nagasaki University.

Molecular karyotyping

DNA oligomicroarray hybridization, using the GeneChip Human Mapping 250K Nsp Array (Affymetrix, Santa Clara, CA, USA), was carried out for 18 patients with KS, following the provided protocol (Affymetrix). Data were analyzed using GTYPE (GeneChip Genotyping Analysis Software) to detect copy number aberration and visualized using CNAG (Copy Number Analyser for GeneChip) version 3.[21] References for non-paired analysis of CNAG were chosen from eight unrelated individuals of HapMap samples from the Affymetrix website (http://www.affymetrix.com/support/). The resolution of this procedure was estimated as ~30–100 kb. CNAG version 3 was linked with the University of California Santa Cruz (UCSC) genome browser (http://genome.ucsc.edu/) assembly May 2004, and then its physical position was referred to the data assembly on March 2006 in the UCSC genome browser after adjustment.

Validation of deletion

Quantitative PCR (qPCR) analysis to validate deletions was run on a Light-Cycler 480 Real-Time PCR System (Roche Diagnostics, Mannheim, Germany) using an intercalating dye, SYTO9 (Molecular probes, OR, USA), which is an alternative to SYBR green I.[22] Absolute quantification was carried out using a second derivative max method. A standard curve of amplification efficiency for each set of primers was generated with a serial dilution of genomic DNA. A corrected gene dosage was given as the ratio of a target gene divided by an internal control gene. The copy number was obtained from a calibration under the assumption that the control genome was diploid.

Target genes of copy number aberration were as follows: *SUMF1* (for patient K9); *MAMDC2* (for patient K16); and *CETN1* (for patient K34). The primer sequences of these genes are available in the online supplementary file. Internal control diploid genes were *OAZ2* and *USP21*. Primer sets of the control genes for genomic DNA were selected from the Real Time PCR Primer Sets website (http://www.realtimeprimers.org/). The control genes were confirmed to have no copy number variants on the Database of Genomic Variants (DGV) updated on 26 June 2008 (http://projects.tcag.ca/variation/). BLAST searches confirmed all primer sequences specific for the gene.

Samples were analyzed in triplicate in a 384-well format in a 10 μl final volume containing about 2 ng genomic DNA, 0.5 μM forward primer, 0.5 μM reverse primer, 0.1 Units TaKaRa ExTaq HS version (TaKaRa, Kyoto, Japan), 1× PCR buffer, 200 μM dNTP and 0.5 μM SYTO9. The amplification conditions consisted of an initial denaturation at 95 °C for 5 min, followed by 45 cycles of

Table 1 Detected genomic copy number aberrations in 17 patients with Kabuki syndrome

Cytoband	*Patient(s) ID*	*CN State*	*Length*	*Physical position*		*Involving gene(s)*	*Concordant loss/gain on DGV*
				Start	*End*		
3p26.3	K7	1	460 kb	1435279	1895554	NR	Variation_8235
3p26.2	K9	1[a]	205 kb	4009368	4214847	*SUMF1*	Variation_8973, 8975, 30169
4q13.2	K23	1[a]	1.26 Mb	66329014	67591611	NR	NR
5q21.2-q21.3	K22	1	281 kb	104301325	104581898	NR	Variation_3568
9q21.11-q21.12	K16	1[a]	1.27 Mb	71760296	73031176	*TRPM3, KLF9, SMC5, MAMDC2*	NR
14q11.2	K5	1	166 kb	19336854	19502641	*OR4N2, OR4K2, OR4K5, OR4K1*	Variation_0376, 7028, 8094, 9234, 9235
15q11.2	K1, K23	1	972 kb	19356830	20329239	*OR4M2, OR4N4, LOC65D137*	Variation_0318, 3070, 8265, 9251, 9254, 9256
18p11.32	K34	1[a]	35 kb	545074	580003	*CETN1*	Variation_5044
20p12.1	K6	1[a]	152 kb	14993412	15145890	*C20orf133 (MACROD2)*[b]	NR
4q12	K5	3	104 kb	54251599	54355281	NR	NR
8q11.21	K7	3	171 kb	50641101	50812548	NR	Variation_2751, 3731, 8601, 37765
10p15.2-p15.1	K5	3	142 kb	3663600	3805292	NR	NR
13q31.1	K6	3	72 kb	82451568	82523728	NR	NR
15q11.2	K7, K9, K12	3	877 kb	19112164	19989036	*CXADRP2, POTEB*	Variation_3070, 3951, 8784, 30670, etc.
15q25.1	K9	3	165 kb	76992181	77156751	*CTSH, RASGRF1*	Variation_3970, 7073
16q21	K13	3	283 kb	58508008	58791285	NR	NR
17q12	K7	3	495 kb	31428390	31923810	*CCL3, CCL4, CCL3L1, CCL3L3, CCL4L1, CCL4L2, TBC1D3B, TBC1D3C, TBC1D3G*	Variation_3142, 4031, 8841, 30824, etc.
22q11.22	K5, K12	3	278 kb	20907806	21186081	*VPREB1, ZNF280B*	Variation_5356, 34540

Abbreviations: CN, copy number; DGV, Database of Genomic Variants; NR, no registration in UCSC genes or DGV.
[a]Validated by quantitative PCR.
[b]Deleted region was within intron 5 of the *C20orf133 (MACROD2)* and did not involve any coding exon.[15]

denaturation at 95 °C for 10 s, annealing at 55 °C for 10 s and extension at 72 °C for 15 s The data were analyzed using LightCycler 480 Basic Software (Roche Diagnostics) and the melting curve was checked to eliminate non-specific products from the reaction.

Mutation screening of candidate genes

Candidate genes, identified within a detected deletion, consisted of four genes: *TRPM3* (NM_001007471 and NM_206946), *KLF9* (NM_001206), *SMC5* (NM_015110) and *MAMDC2* (NM_153267) located at 9q21.12–q21. 11. The entire coding region and splice junctions of the genes were sequenced on an automated sequencer 3130xl (Applied Biosystems, Foster City, CA, USA) using BigDye version 3.1 (Applied Biosystems). Genomic sequences were retrieved from the UCSC genome browser (assembly: March 2006). PCR primers were designed with the assistance of Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi). The primer sequences are available in the online supplementary file. Resultant electropherograms were aligned using ATGC version 3.0 (Software Development, Tokyo, Japan) and inspected visually to find DNA alterations.

In silico analysis

Relations among deleted genes were assessed using online software, PANTHER (Protein Analysis Through Evolutionary Relationships, http://www.pantherdb.org), to determine whether the genes involve some developmental pathway or biological process.[23] The novel synonymous base substitutions found in the mutation screening were examined for their potential activation of the cryptic splice site by comparison between wild-type allele and mutated allele using the GeneSplicer program (http://www.cbcb.umd.edu/software/GeneSplicer/gene_spl.shtml).

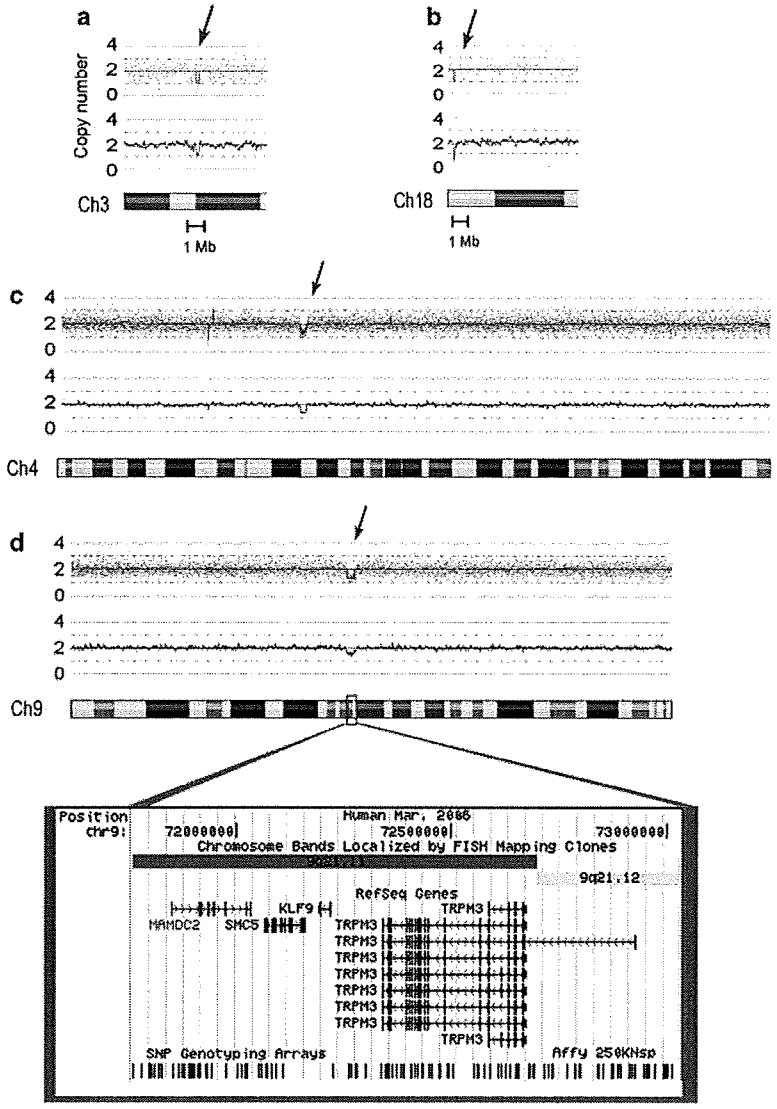

Figure 1 Chromosome view of Copy Number Analyser for GeneChip (CNAG) analysis. Each dots represent fluorescent intensity on each single-nucleotide polymorphisms (SNP) probe of GeneChip 250K NspI array (Affymetrix). Solid lines indicate copy number analyzed with CNAG. Arrows show detected deletions. (**a**) Chromosome (Ch) 3 of patient K9, ~205 kb deletion in 3p26.2 involving an exon of *SUMF1*gene. (**b**) Chromosome 18 of patient K34, ~35 kb deletion in 18p11.32, containing the *CETN1* gene. (**c**) Chromosome 4 of patient K23, ~1.26 Mb deletion in 4q13.2, not involving any known gene. (**d**) Chromosome 9 of patient K16, ~1.27 Mb deletion in 9q21.11–q21.12, harboring four genes: *TRPM3*, *KLF9*, *SMC5* and *MAMDC2*. The University of California Santa Cruz genome browser denotes the cytobands, genes and probe setting of Affymetrix 250K NspI array within the region. No copy number variation was registered here in the Database of Genomic Variants updated 26 June 2008. FISH, fluorescent *in situ* hybridization.

RESULTS

Molecular karyotyping and validation of deletion

The entries of molecular karyotyping were 18 patients with KS (K1, K3, K5, K6, K7, K8, K9, K11, K12, K13, K16, K18, K20, K21, K22, K23, K34 and K38). We eliminated the data of patient K3 from copy number analysis, because it showed low quality data; that is, a single-nucleotide polymorphism (SNP) call rate of 82.51% and a quality control performance detection rate of 74.09%, probably because of DNA degradation during long-term storage. The other patients showed high call rates, enough for copy number analysis (SNP call rate of 90.07–97.72% and detection rate of 91.52–99.77%). We identified nine deleted regions, the lengths of which were between ~35 kb and ~1.27 Mb, and nine duplicated regions, of lengths between ~72 and ~495 kb, in the 17 patients analyzed (Table 1). As for the nine duplications detected, five of them were concordant to several observed gains in DGV, and four of them in each patient did not contain any known genes.

It is interesting that the deleted region of 9q21.11–q21.12 (~1.27 Mb in patient K16), which had not been registered in DGV, harbored four known genes: transient receptor potential cation channel, subfamily M, member 3 (*TRPM3*), Kruppel-like factor 9 (*KLF9*), structural maintenance of chromosomes protein 5 (*SMC5*) and MAM domain containing 2 (*MAMDC2*) (Figure 1d). The deletion of 3p26.2 (~205 kb in patient K9, Figure 1a) had involved a non-coding exon of the *SUMF1* gene. The deletion of 18p11.32 (~35 kb in patient K34, Figure 1b) containing the *CETN1* gene had one registration in DGV as Variation_5044, which described only one observed loss and 14 observed gains in 95 individuals. The deletion of 4q13.2 (~1.26 Mb in patient K23, Figure 1c) and 20p12.1 (~152 kb in patient K6) did not carry any coding exon of any gene. The regions of 14q11.2 (~116 kb in patient K5) and 15q11.2 (~972 kb in patient K1 and K23) were non-pathological deletions with as many registrations as observed losses in DGV.

To validate the deletion of the detected region, we confirmed the loss of heterozygosities of the SNP probes present there using GTYPE (data not shown) and carried out qPCR. The regions of *SUMF1* on 3p26.2 (for patient K9) and of *MAMDC2* on 9q21.11–q21.12 (for patient K16) had one copy in each patient compared with those in unaffected individuals (Figure 2). The deletion of *CETN1* on 18p11.32 (for patient K34) was inherited from his unaffected mother. As samples from the parents of patient K16 were unavailable, it was not possible to examine whether the deletion of 9q21 was *de novo*. But the deletion was not found in 95 normal Japanese individuals using qPCR (data not shown).

As a consequence of this copy number analysis, we considered the next four genes as candidate genes for KS: *TRPM3*, *KLF9*, *SMC5* and *MAMDC2*.

Mutation screening and *in silico* analysis

Table 2 shows the results from mutation screening of the four candidate genes in 41 patients with KS. Ten base substitutions were found in the 41 patients, consisting of six registered SNPs, one unregistered SNP and three silent mutations. In addition, *SUMF1* (NM_182760) and *CETN1* (NM_004066) were also screened, but no mutations were detected (data not shown).

We checked the three silent mutations for splice site alteration using the GeneSplicer program, but no activation of the cryptic splice site was predicted. Although PANTHER classification of the four candidate genes did not show significant correlation for biological processes or pathway because of its small scale in number, some genes associated with developmental biology; that is, DNA repair (*SMC5*) and mRNA transcription regulation (*KLF9*).

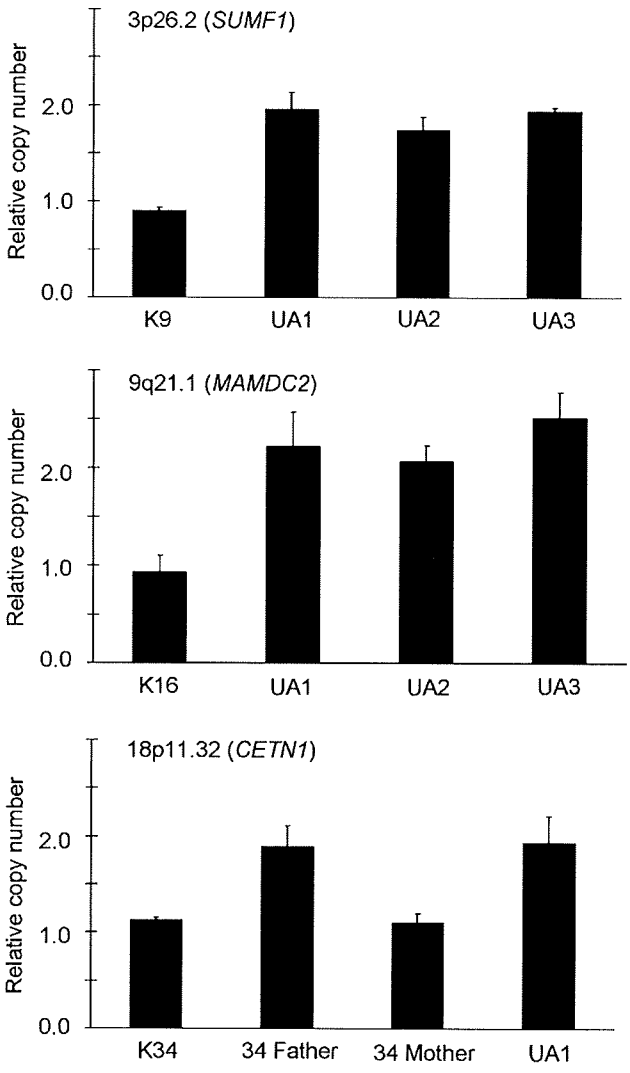

Figure 2 Validation of deletion with quantitative PCR (qPCR). qPCR confirmed a loss of one copy in each patient: *SUMF1* at 3p26.2 for patient K9; *MAMDC2* at 9q21.1 for patient K16; *CETN1* at 18p11.32 for patient K34. The deletion of patient K34 was inherited from his unaffected mother. UA, unaffected individual. Error bars, s.d.

DISCUSSION

We used high-resolution oligonucleotide array of GeneChip 250K NspI with a resolution of 30–100 kb and tried to find causative deletions or mutated genes for KS. Our molecular analysis did not strongly identify the causative gene for KS, but we identified a locus that possibly contributed to KS.

The deletion in patient K16, with a length of ~1.27 Mb at 9q21.11–q21.12, harbored four known genes: *TRPM3*, *KLF9*, *SMC5* and *MAMDC2* (Figure 1d). Unfortunately, her parents' DNAs were unavailable, but the region is unlikely to be a copy number variant (CNV) because it has not been known as CNV in DGV; moreover, the deletion was not found in 95 normal Japanese individuals using qPCR.

As mutation screening in the 41 patients with KS showed no pathogenic base substitution in these genes, we cannot state that

Table 2 Mutation screening of candidate genes in 41 patients with Kabuki syndrome

Involving gene(s)	*Base substitution*	*Amino acid change*	*Patient(s) with KS*		*dbSNP*	*Allele frequency among unaffected Japanese*[a]	*Results of mutation screening*
			Homo	*Hetero*			
TRPM3	459C>T	A153A	0	1	NR	0	Synonymous
	4023G>A	S1341S	13	28	rs3739776	—	SNP, synonymous
KLF9	459C>T	V153V	0	1	NR	0	Synonymous
SMC5	916G>A	V306I	37	4	rs1180116	—	SNP, non-synonymous
	922T>C	C308R	21	10	rs1180117		SNP, non-synonymous
MAMDC2	62T>C	L21P	0	2	NR	0.02	SNP, non-synonymous
	492C>T	T164T	0	1	NR	0	synonymous
	816C>T	Y272Y	11	16	rs2296772	—	SNP, synonymous
	867G>A	A289A	13	15	rs2296773	—	SNP, synonymous
	1063_1065 delAAA	K355 del	11	17	rs61609258	—	(SNP) synonymous; del/ins polymorphism

Abbreviations: *KLF9*, Kruppel-like factor 9; *MAMDC2*, MAM domain containing 2; *SMC5*, structural maintenance of chromosomes protein 5; SNP, single nucleotide polymorphism; *TRPM3*, transient receptor potential cation channel, subfamily M, member 3; dbSNP, registration number of database of SNP (http://www.ncbi.nlm.nih.gov/SNP/).
[a]Allele frequency was calculated from 188 chromosomes of 94 individuals.

these genes are major genetic factors for KS. However, it is presumable that the genes have some etiological roles for KS because of its genetic heterogeneity. Ontology of the PANTHER classification suggested that the three genes were associated with developmental biology, such as mRNA transcription regulation. Moreover, the 1.27 Mb region of 9q21 was included in an earlier reported candidate locus of cleft lip/palate by meta-analysis of linkage analysis.[24] Patient K16 actually had velopharyngeal insufficiency because of a submucous cleft palate. Therefore, it is reasonable to consider that the deleted genes cooperated with the development of a cleft palate, which is often accompanied by KS.

Although the ~152 kb deletion within intron 5 of *C20orf133 (MACROD2)* in patient K6 did not involve any coding exon and her parents' DNAs were unavailable, the deletion was neither registered as CNV in DGV nor was it found in 95 normal Japanese individuals by qPCR (data not shown). Maas *et al.*[14] reported *de novo* ~250 kb deletion, including exon 5 of *C20orf133 (MACROD2)*, in a patient with KS. Direct sequencing for the gene in 62 other patients with KS did not detect mutations,[14,15] but the gene may be one of the causative genes for KS in consideration of its genetic heterogeneity.

We focused this study on KS on deletion/duplication detected using oligonucleotide array and mutation screening of the coding genes within the region. One limitation of this study is its resolution. As a matter of course, a higher resolution array can detect smaller genomic rearrangements, which were undetectable in the same patient, as we showed here compared with an earlier study of BAC array CGH.[18] Although SNP probes are useful to examine loss of heterozygosity as a collateral evidence in deletions, unevenly distributed probes of the SNP array have a disadvantage for CNV detection. As various platforms on oligonucleotide array have developed, higher resolution platforms will have to be applied to search tiny genomic rearrangements in patients with KS. Another limitation is that we assumed that a single copy number change caused KS. It remains to be elucidated whether CNV association[25] contributes towards manifestations of KS. If further investigation with refined array technologies cannot find the etiology of KS, the direction of study for KS will have to be changed to find *de novo* sequence alteration or methylation aberration, including in the non-coding genomic regions.

In summary, we applied molecular karyotyping with GeneChip 250K array to detect copy number aberrations in 17 patients with KS and screened four candidate genes in 41 patients with KS. We could not identify causative DNA alteration for KS, but the locus, 9q21.11-q21.12, including *TRPM3*, *KLF9*, *SMC5* and *MAMDC2*, may contribute to the cleft palate of KS. Further investigations will be needed as various array platforms have the potential to specify genomic alterations for KS.

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The FASEB Journal • Research Communication

Earwax, osmidrosis, and breast cancer: why does one SNP (538G>A) in the human ABC transporter *ABCC11* gene determine earwax type?

Yu Toyoda,* Aki Sakurai,*,† Yasumasa Mitani,†,‡ Masahiro Nakashima,§ Koh-ichiro Yoshiura,‖ Hiroshi Nakagawa,* Yasuo Sakai,¶ Ikuko Ota,†,# Alexander Lezhava,† Yoshihide Hayashizaki,† Norio Niikawa,‖, and Toshihisa Ishikawa*,†,1**

*Department of Biomolecular Engineering, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Yokohama, Japan; †Omics Science Center (OSC), RIKEN Yokohama Institute, Yokohama, Japan; ‡K.K. DNAFORM, Yokohama, Japan; §Tissue and Histopathology Section, Atomic Bomb Disease Institute, and ‖Department of Human Genetics, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan; ¶Department of Plastic and Reconstructive Surgery, Fujita Health University, Toyoake, Japan; #Department of Gastroenterological Surgery, Yokohama City University Graduate School of Medicine, Yokohama, Japan; and **Research Institute of Personalized Health Sciences, Health Sciences University of Hokkaido, Hokkaido, Japan

ABSTRACT **One single-nucleotide polymorphism (SNP), 538G>A (Gly180Arg), in the *ABCC11* gene determines the type of earwax. The G/G and G/A genotypes correspond to the wet type of earwax, whereas A/A corresponds to the dry type. Wide ethnic differences exist in the frequencies of those alleles, reflecting global migratory waves of the ancestors of humankind. We herein provide the evidence that this genetic polymorphism has an effect on the *N*-linked glycosylation of ABCC11, intracellular sorting, and proteasomal degradation of the variant protein. Immunohistochemical studies with cerumen gland-containing tissue specimens revealed that the ABCC11 WT protein was localized in intracellular granules and large vacuoles, as well as at the luminal membrane of secretory cells in the cerumen gland, whereas granular or vacuolar localization was not detected for the SNP (Arg180) variant. This SNP variant lacking *N*-linked glycosylation is recognized as a misfolded protein in the endoplasmic reticulum and readily undergoes ubiquitination and proteasomal degradation, which determines the dry type of earwax as a mendelian trait with a recessive phenotype. For rapid genetic diagnosis of axillary osmidrosis and potential risk of breast cancer, we developed specific primers for the SmartAmp method that enabled us to clinically genotype the *ABCC11* gene within 30 min.—Toyoda, Y., Sakurai, A., Mitani, Y., Nakashima, M., Yoshiura, K., Nakagawa, H., Sakai, Y., Ota, I., Lezhava, A., Hayashizaki, Y., Niikawa, N., Ishikawa, T. Earwax, osmidrosis, and breast cancer: why does one SNP (538G>A) in the human ABC transporter *ABCC11* gene determine earwax type? *FASEB J.* 23, 2001–2013 (2009)**

Key Words: endoplasmic reticulum-associated degradation · genetic polymorphism · N-linked glycosylation · protein instability

CELL SECRETION IS AN IMPORTANT physiological process that ensures smooth metabolic activities and tissue repair as well as growth and immunological functions in the body. Human earwax is a dimorphic trait consisting of wet or dry types. Wet earwax (cerumen) is a secretory product of ceruminous apocrine glands, whereas the dry type of earwax lacks oily components. In addition, apocrine glands can be found in the external auditory canal, breast, and axillary region; those physical characteristics also are concerned with apocrine glands. A positive association among the wet earwax type, axillary osmidrosis (1), colostrum secretion (2), and breast cancer risk (3, 4) has been suggested by phenotype-based analysis. Apocrine secretion occurs when the secretory process is accomplished with a partial loss of cell cytoplasm. The secretory materials may be contained within the secretory vesicles or dissolved in the cytoplasm, and during secretion they are released as cytoplasmic fragments into the glandular lumen or interstitial space (5).

Hitherto apocrine secretory mechanisms have not been well characterized (5). Although the biochemical and physiological pathways that regulate the apocrine secretory process are not clearly known, our recent finding that the nonsynonymous SNP 538G>A (rs17822931; Gly180Arg) in the *ABCC11* gene determines the type of earwax has shed light on the novel function of this ABC transporter in apocrine glands (6). In 2001, we and other groups independently cloned human ABCC11 and ABCC12 cDNAs (7-9). Both *ABCC11* and *ABCC12* genes are located on human chromosome 16q12.1 in a tail-to-head orientation with a separation distance of ~20 kb (7). Interestingly, there is an orthologous gene corresponding to

[1] Correspondence: Department of Biomolecular Engineering, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259-B-60 Nagatsuta, Midori-ku, Yokohama 226-8501, Japan. E-mail: tishikaw@bio.titech.ac.jp

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