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O-06: アレイ染色体検査のための健常人 CNV データベース構築の試み. 松井 健, 霜川 修, 吉浦孝一郎, 新川詔夫, 松本直通, 原田直樹

O-08: Brain AVM maps to 5p13-q14, 15q11-q13 or 18p11: Linkage analysis with clipped fingernail DNA using high-density SNP array. 及川将弘, 国場英雄, 近藤達郎, 永安 武, 新川詔夫, 吉浦孝一郎

O-11: PCR-高解像度融解曲線分析法による遺伝子スキャンニングシステムの構築. 要 匡, 柳 久美子, 福嶋義光, 吉浦孝一郎, 新川詔夫, 成富研二

O-12: 次世代型シーケンサを用いた原因候補全領域リシーケンス解析へのアプローチ:日本人ゲノム 16q122 領域の構造解析. 要 匡, 塚原正俊, 柳 久美子, 藤森一浩, 喜久里育也, 照屋盛実, 今田有美, 鼠尾まい子, 矢野修一, 佐藤友紀, 三輪有希乃, 平野 隆, 平野隆城, 高嶋 博, 吉浦孝一郎, 新川詔夫, 成富研二

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PB 148: 嚢胞内乳ガンは嚢胞内乳頭腫に比べ顕著なゲノム変化を有する:高密度 SNP マイクロアレイによるゲノムワイド copy number/LOH 解析. 及川将弘, 永安武, 矢野洋, 安倍邦子, 林徳真吉, 新川詔夫, 吉浦孝一郎

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H. 知的財産権の出願・登録状況（予定を含む。）

1. 特許得取得

   なし

2. 実用新案登録

   なし

3. その他

   なし

厚生労働科学研究費補助金（難治性疾患克服研究事業）
平成２１年度分担研究報告書

研究課題：ゲノム異常症として歌舞伎症候群原因遺伝子同定と遺伝子に基づく成長障害治療可能性の研究開発

分担研究項目：マッピング，候補遺伝子解析，変異解析
分担研究者：木下　晃（長崎大学大学院医歯薬学総合研究科
人類遺伝学・助教）

研究要旨
本分担研究研究の目的は（1）未だ原因不明である歌舞伎症候群（新川-黒木症候群）の原因遺伝子の同定と病態生理の解明である。本年度の分担研究として，これまでに得ていた患者試料の欠失部分の遺伝子変異解析と 2.7M array による全ゲノムコピー数異常の検出を行った。

A．研究目的

歌舞伎症候群（新川-黒木症候群）の原因遺伝子の同定と病態生理の解明を目指して本年度は，これまでに解析し明らかとなっていた，患者の欠失領域に含まれる遺伝子の変異解析と 2.7M array 解析を行った。

B．研究方法

北海道医療大学の研究分担チームから供給された 63 名の歌舞伎症候群患者試料を使用して，2.7M array によりコピー数異常を検出する。また，これまでに明らかにしてきた欠失部位を定量 PCR 法により確定させる。

(1) Genome 2.7M Array を用いての全ゲノム微少コピー数異常領域の同定

患者試料から抽出された高分子 DNA を使用する。Cytogenetics Whole - Genome 2.7M Array は Affymetrix 社から供給されるプロトコールに則り実験し，データ解析は Partek Genomics Suite および Chromosome Analysis Suite (ChAS) Software を使用する。

(2) 候補遺伝子の変異解析・定量解析

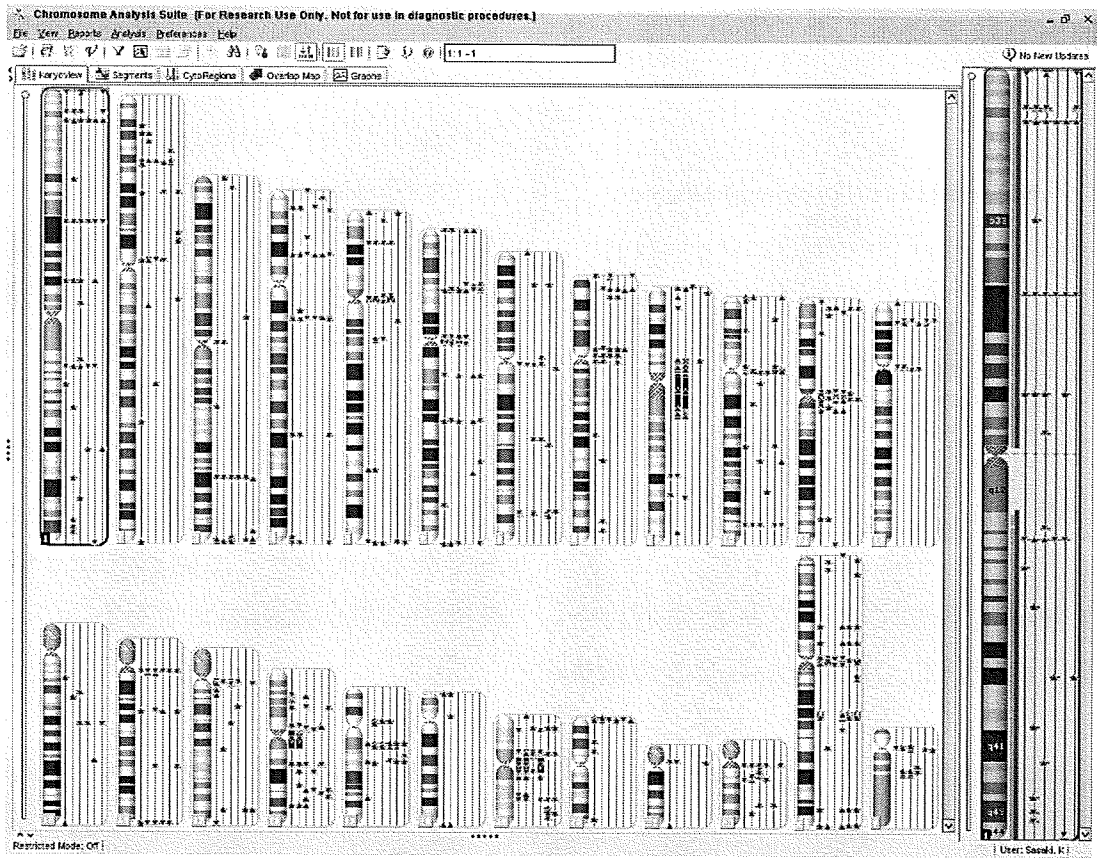
かねてから口唇のくぼみ（lip pit）が Van der Woude 症候群（VWS）

に特徴的とされていたが，歌舞伎症候群の一症状として見られることが知られていて，VWS の原因である IRF6 遺伝子の情報伝達系に関する遺伝子が原因であるかもしれないとの仮説に基づいて IRF6 関連遺伝子の変異解析・定量解析を行う。変異解析は従来からの PCR-direct sequence 法を採用し，定量は，SYTO13 による増幅率によってコピー数の定量を行う。PCR の機械は Roche 社の lightCycler480 を使用する。本年度中に，定量 PCR 時の反応液作成や分注作業は特化した分注機（Qiagen 社の Qiagility）を使用することによって，安定したデータがえられることがわかり，Qiagility 購入し実験することとした。

以前，我々は 9q21.11-q21.12（～1.27 Mb)の欠失が，遺伝子を含んでかつコピー数多型の報告がない場所として報告した（Kuniba et al., 2009)。その欠失部位なかには，TRPM3, KLF9, SMC5, MAMDC2 の４遺伝子が欠失領域に含まれていたので，このなかの遺伝子の変異解析を行った。

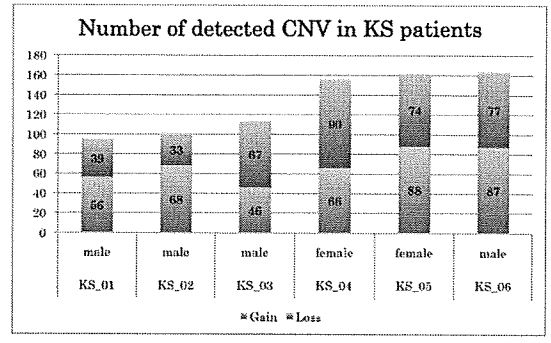
C. 研究結果

Cytogenetics Whole - Genome 2.7M Array が供給され始めたので，それを用いて，まずは６例をパイロットスタディーとしてコピー数変化の同定を行った。



（図１）

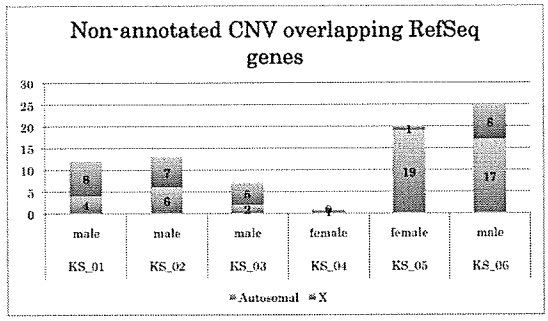
染色体の模式図の右側に６人分の結果が表示されている。赤い下方を向いた▼が欠失箇所で，青い上方を向いた▲が重複箇所である。右に第一番染色体を拡大した図が表示されている。６名の患者解析の結果をそれぞれの患者について欠失および重複の数を表示したのが表１である。



（表１）

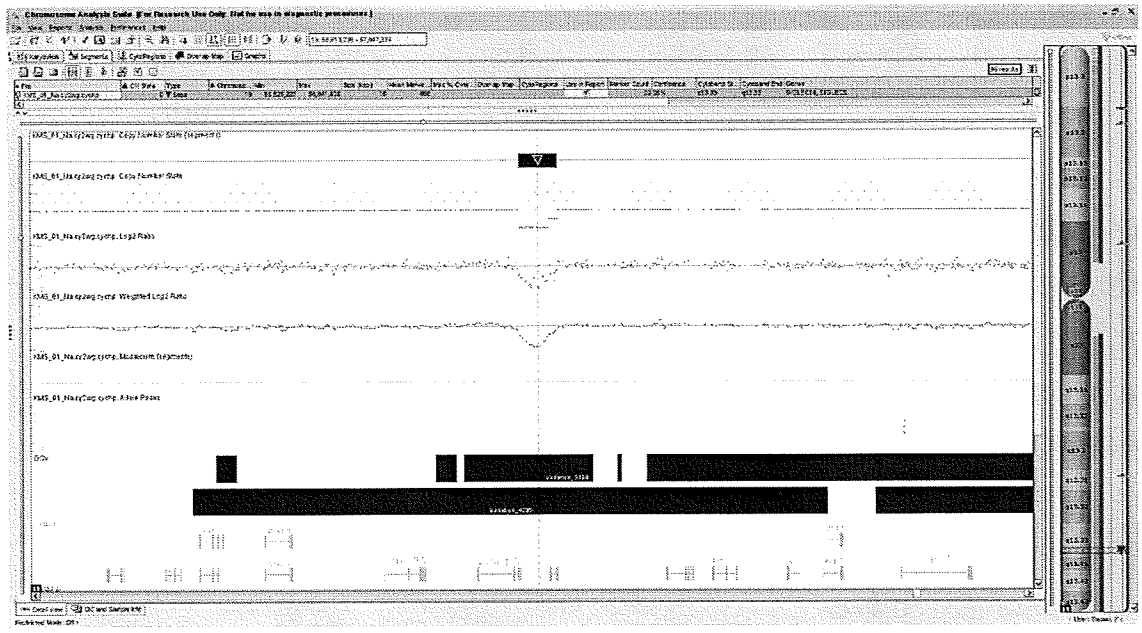
１名につきおよそ１００カ所のコピー数変化が見られる。このうち，歌舞伎症候群の原因として可能性があるのは，多型としてデータベースに登録されておらず，かつ遺伝子領域を含んでいる部位である。そのような部位を

抽出し数を表示したのが次の表２である。



（表２）

候補領域として選択された領域は各個人において，１カ所から２５カ所まで様々であった。また，既存の公開データベース以外に自ら解析した対照試料および affymetrix 社から提供されている対照試料にもない領域が３カ所残った。２カ所が重複で３コピー，１カ所が欠失である。３カ所に７遺伝子が含まれている。現在７遺伝子については，SYTO13 を使った定量 PCR 法によってコピー数状態を確認しつつ，他の患者で変異解析を行っているところである。欠失として確認された Chip 解析の結果が次の図２である。



（図２）

図２の例では，図の中央に欠失領域が描出されている。欠失範囲は 19 kb であった。

以前発見していた欠失部位なかには，TRPM3，KLF9，SMC5，MAMDC2の４遺伝子が欠失領域に含まれていた。これらの遺伝子について，他の患者の

DNAを用いて変異解析を行ったが，原因変異と判断できる変異はなかった。イギリスから，歌舞伎症候群様の患者で9q21.11付近に転座を認めて，転座の切断点がTRPM3遺伝子の中にある症例の相談を受け，その患者のTRMP3遺伝子を解析した。しかし，変異は認められなかった。TRMP3遺伝子の変異が歌舞伎症候群様の症状をもたらす可能性があるが確定できない。

D. 考察

Affymetrix Cytogenetics Whole - Genome 2.7M Array の供給が遅れたが，今後は，当初の予定通り，2.7M Array を使って新規歌舞伎典型例と既試料所有の合計63例を対象として，Cytogenetics Whole - Genome 2.7 M Arrayにより解析を進める。解像度は10ー20 kbと考えられ，エキソン単位での欠失・重複が検出できると考えられる。歌舞伎症候群が単純な機能喪失による疾患であれば，63例中に欠失が認められるであろうし，原因は見つけられる。

E. 結論

2.7M array の解析は，消耗品の供給が年度末近くになったために，まだ進んでいないが，確実に解析を進めスクリーニングが完了できるであろう。

array解析による構造異常同定を補う意味でのexome解析を今後予定している。exome 解析は，本年度に研究グループとして次世代型シーケンサーの使用実績を積んでおり来年度に向けての準備は完了している。

――達成度について――

主たる目標であった Cytogenetics Whole - Genome 2.7 M Array により解析が遅れている。これまで描出していた 9q21.11 欠失，8q24.11 の欠失の他の患者での確認，およびこれらの欠失領域に見られる遺伝子の変異解析に時間を取られた。年度終了間際になり 2.7 M Array が利用可能となって，解析を進めている最中である。これまでの結果の検証は全て終了し，研究計画していた実験が始まった段階である。既所有のLCL細胞についても新たに解凍し培養し高品質のDNAを揃え今後のどのような解析にも耐えうるDNAの確保を行った。Cytogenetics Whole - Genome 2.7 M Array 解析をできる限り進め，欠失解析の目標達成をはかる。

F. 健康危険情報

なし

G. 研究発表

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H. 知的財産権の出願・登録状況(予定を含む。)

1. 特許得取得
   なし
2. 実用新案登録
   なし
3. その他
   なし

III. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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IV. 研究成果の刊行物・別冊

Journal of Human Genetics (2009), 1–6

www.nature.com/jhg

ORIGINAL ARTICLE

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

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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.
Journal of Human Genetics advance online publication, 3 April 2009; doi:10.1038/jhg.2009.30

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

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Received 15 January 2009; revised 3 March 2009; accepted 11 March 2009

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 LightCycler 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).

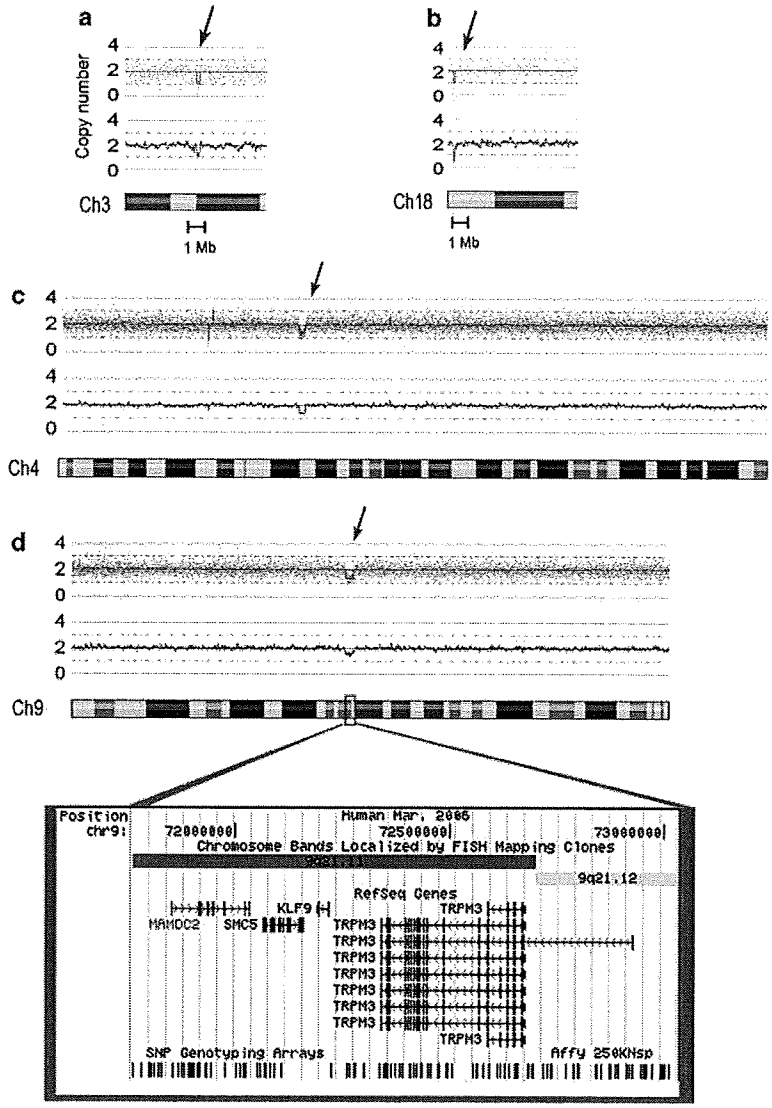

Figure 1 Chromosome view of Copy Number Analyser for GeneChip (CNAG) analysis. Each dots represent fluorescent intensity on each single-nucleotide polymorphisms (SNP) probe of GeneChip 250K NspI array (Affymetrix). Solid lines indicate copy number analyzed with CNAG. Arrows show detected deletions. (**a**) 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*).

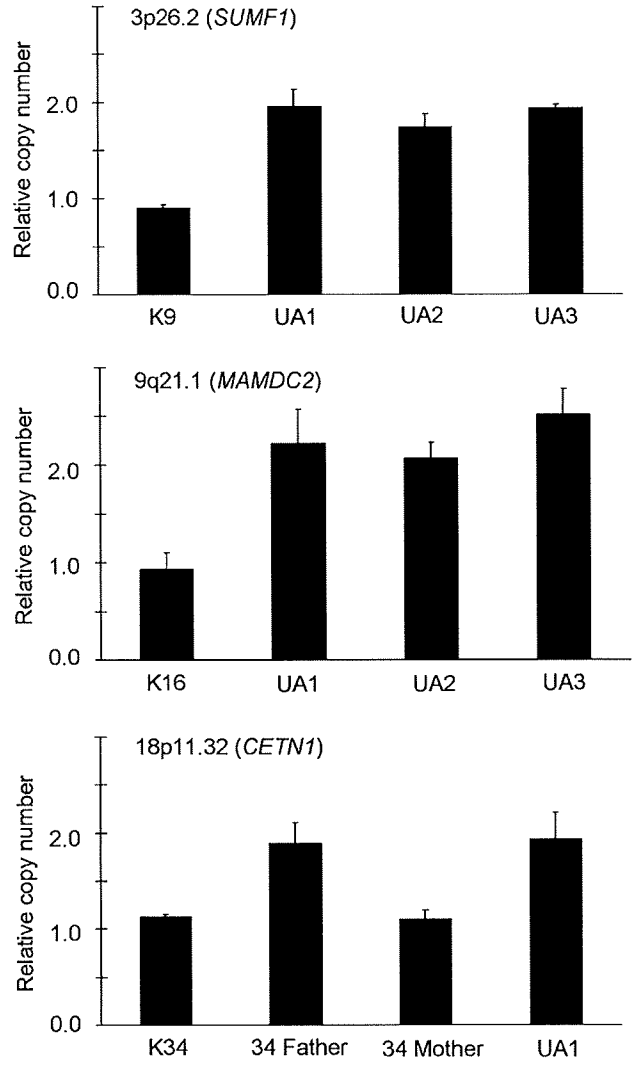

Figure 2 Validation of deletion with quantitative PCR (qPCR). qPCR confirmed a loss of one copy in each patient: *SUMF1* at 3p26.2 for patient K9; *MAMDC2* at 9q21.1 for patient K16; *CETN1* at 18p11.32 for patient K34. The deletion of patient K34 was inherited from his unaffected mother. UA, unaffected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

ACKNOWLEDGEMENTS

We are grateful to the patients and their parents for their participation in this research. We also thank Ms Yasuko Noguchi, Ms Miho Ooga and Ms Chisa Hayashida for their technical assistance. NN was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Sports, Culture, Science and Technology of Japan, and was supported by SORST from Japan Science and Technology Agency (JST) (Nos. 17019055 and 19390095, respectively). KY was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Health, Labor and Welfare.

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