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２２年度分担研究報告書

研究課題：ゲノム異常症としての歌舞伎症候群原因遺伝子同定と遺伝子情報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，候補遺伝子解析および変異解析
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）
研究協力者：要　匡（琉球大学大学院医学研究科・遺伝医学・准教授）
：松本直通（横浜市立大学大学院・遺伝学・教授）

研究要旨
本分担研究研究の目的は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。北海道医療大学と Washington University の共同研究による exome 解析の結果，MLL2 遺伝子変異が歌舞伎症候群の原因の原因であることが判明した。本年度の分担研究として，日本人歌舞伎症候群患者において MLL2 遺伝子の変異検出を行い，phenotype-genotype correlation を明らかとすることを目的とした。

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，新技術である次世代型シーケンサーを用いた exome 解析により，原因遺伝子を同定することを目的とした。

B. 研究方法

臨床遺伝医により診断確実な歌舞伎症例でかつ Washington University で解析していない８例を対象として，長崎大学で変異解析を行った。

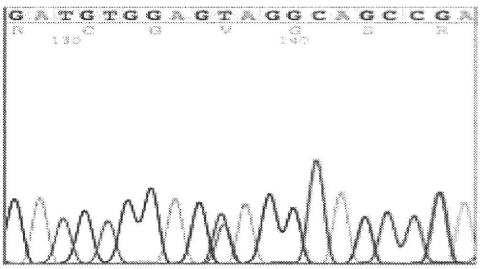
変異解析のための PCR 用プライマーの塩基配列は，研究協力者である琉球大学医学部の要匡より情報提供してもらった。MLL2 遺伝子の全 54 エキソンを exon-intron 境界領域も含め増幅し塩基配列決定をおこなった。

C. 結果

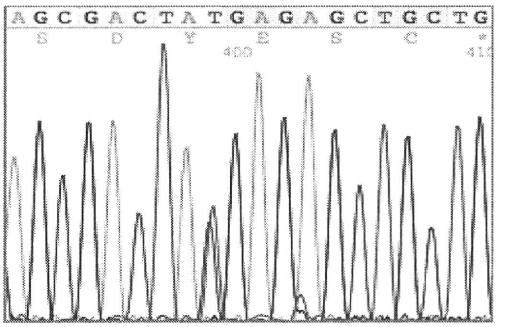
MLL2 の変異は，北海道医療大学グループと Washington University グループとによって，歌舞伎症候群患者の約

80 % に認められ，MLL2 の truncation type 変異すなわち機能喪失をもたらす変異が歌舞伎症の原因となっていることが明らかとなっている。

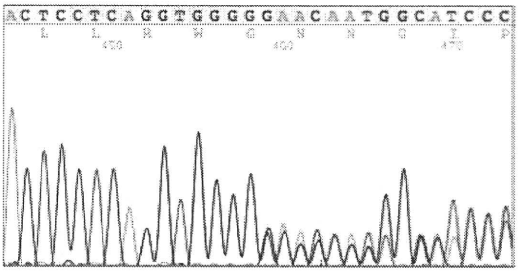
今回はまだ介せｋしていない８例を解析し，１例で１塩基欠失（frameshift），２例で nonsense mutation，１例でエキソンの最終塩基の G to A transition，１例でアルギニンからグルタミンへの missense mutation が見つかった。3 例は塩基変異を確認できなかった。



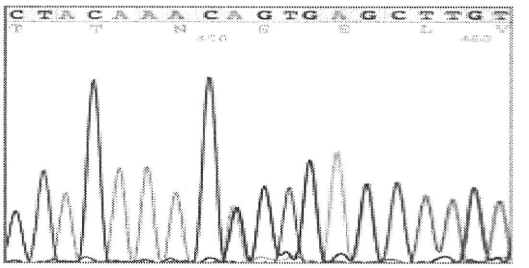
Nonsense 変異 1



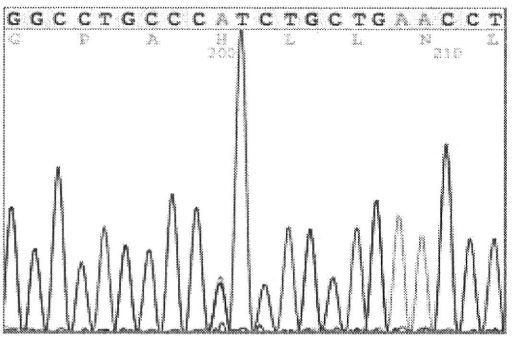
Nonsensne 変異 2



One base deletion



Exon の最終塩基 G->A 変異



CAT->CGT missense 変異

D. 考察

今回の研究でMLL2変異が歌舞伎症候群の原因の一つとなっていることは明らかである。70～80%の患者において完全長のタンパク質が合成されないtruncation型の変異が検出され

る。しかし，残りの20～30%の患者にはMLL2の変異が認められず，他の遺伝子変異によって歌舞伎症候群が発症していることが考えられる。来年度から，MLL2遺伝子変異で説明不可能な症例の原因追及が研究課題として加わる。

歌舞伎症候群の原因が判明したが，歌舞伎症候群の多様な臨床症状とMLL2の変異部位・変異の種類との明確な関連，すなわちphenotype-genotype correlationは明らかでない。今回解析した８症例も，nonsense 変異１（エキソン17），nonsense 変異１（エキソン32），一塩基欠失（エキソン10），エキソンの最後の塩基変異（エキソン50：おそらくsplice site mutation），missense変異（エキソン48）と遺伝子全体にわたって変異が見つかるのは，最初の報告と変わりはない。また，顔貌から典型的な歌舞伎症候群と思われる患者であっても３名はMLL2変異陰性であった。本年度の変異解析は８人と限られた人数で行ったが，今後はWashington University へ解析を依頼している症例を除いた約20例の既収集試料を用いて変異解析を精力的に進め，phenotype - genotype correlation を明らかにする必要がある。来年度へ向けて日本全国の主な公立病院への調査によって，歌舞伎症候群患者のDNA診断と患者数の把握，phenotype - genotype correlation を明らかとするように研究を進める。

E. 結論

exome解析によって歌舞伎症候群の原因遺伝子の一つ MLL2 遺伝子を明らかにした。次世代型シーケンサーによる原因不明の遺伝子疾患の解析は非常に有用であることが示された。特に，歌舞伎症候群は，次世代型シーケンサー＋exome 解析によって原因遺伝子が明らかにされた最初の常染色体優性疾患である。

最初の Cytogenetics Whole - Genome 2.7 M Array により欠失部位を描出してから遺伝子を同定しようとした試みは失敗し，exome 解析によって MLL2 遺伝子を単離できた。欠失部位を丹念に解析してからの原因遺伝子単離は有用な方法であるが，歌舞伎症候群はほとんど全てが塩基レベルの変異あるいは insertion/deletion であり，欠失部位探索では全く有効性を見出せなかった。結局，疾患によって遺伝子変異の特徴があり，array 解析を行う意味は疾患単位毎に異なることが明らかとなった。

顔貌から典型的歌舞伎症候群であっても MLL2 遺伝子変異がある群とない群が存在している。まだ，phenotype - genotype correlation は明らかではない。

——達成度について——

歌舞伎症候群の原因遺伝子単離を第一の目標として研究を開始し，本年度実その一つとして MLL2 遺伝子を単離できたことは大きな進展である。本年度は，時間が限られていて，多数の歌舞伎症候群患者の DNA 解析を行っていずに，phenotype-genotype correlation を明らかに出来ていない。今後（最終年度）は，全国調査によって歌舞伎症候群（様）の頻度を明らかにすると共に，MLL2 を対象として DNA 診断を行う。phenotype-genotype correlation を明らかにしながら，MLL2 遺伝子変異のない患者について，再度 exome 解析を行う。

F. 健康危険情報

なし

G. 研究発表

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P-024: ABCC11 expression and 538G/A polymorphism in human breast cancer. Sosonkina Nadiya, Starenki Dmytro, 太田　亨，吉浦孝一郎，新川詔夫

P-025: 乳癌FFPE標本を用いたAgilent SurePrint G3 microarray によるアレイCGHの最適化. 及川将弘，蔵重智美，三浦史郎，中島正洋，永安 武，吉浦孝一郎

P-028: Genetic polymorphism of human ABCC11 as a determinant of earwax type, axillary osmidrosis, and the risk of breast cancer. 豊田　優，櫻井亜季, 太田郁子, 坂井靖夫, 五味常明，中川 大，アレキサンダーレジャバ，中島正洋，吉浦孝一郎，林崎良英，新川詔夫，石川智久

P-123: 母体血中における胎盤特異的microRNA群の網羅的スクリーニング. 東島 愛，三浦清徳，三浦生子，山崎健太郎，阿部修平，城　大空，長谷川ゆり，中山大介，木下　晃，吉浦孝一郎，増崎英明

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2-1：アレイCGHを用いたヒバクシャン乳癌のゲノム不安定性の検討. 及川将弘，吉浦孝一郎，蔵重智美，三浦史郎，中島正洋

第48回日本婦人科腫瘍学会（つくば国際会議場、茨城）2010年7月8-10日

妊娠中の子宮頸部細胞診における日母分類、ベセスダシステムおよびHPVスクリーニングの比較. 三浦清徳、山崎健太郎、池本理恵、三浦生子、嶋田貴子、濱口大輔、小寺宏平、藤下晃、鮫島哲郎、村上誠、中山大介、吉浦孝一郎、増崎英明

第34回日本口蓋裂学会総会・学術集会
2010年5月27日（木）～28日（金），北とぴあ，東京

シンポジウムI テーマ：口唇裂・口蓋裂の分子遺伝学研究 –これまでの研究成果とこれからの原因追求 -S I -基調講演: 比較的ありふれた病気（sub-common disease）としての口唇列・口蓋裂. 吉浦孝一郎

第106回　日本精神神経学会学術総会
2010年5月20日（木）～22日（土）
広島国際会議場, 広島

2-F-18: 統合失調症および自閉症一卵性双生児不一致例におけるゲノム構造変化の検証. 小野慎治, 今村 明, 橋田あおい，黒滝直弘，田崎真也，小澤　寛樹，吉浦孝一郎

第110回日本外科学会総会　2010年4月8日（木）～10日（土），名古屋国際会議場，名古屋

PD-9-1: 乳腺乳頭状腫瘍の臨床病理学的特徴と細胞遺伝学的プロファイル. 及川将弘, 吉浦孝一郎, 矢野 洋, 安倍邦子, 林徳真吉, 永安　武

国際学会

2010/10/10-14　20th ISUOG World Congress: (Prague, Czech Republic)

A case of mesenchymal diaplasia. Miura K, Yamasaki K, Miura S, Nakayama D, Yoshiura K, Nakayama M, Masuzaki H

The American Society of Human Genetics, 59th Annual Meeting
Washigton D.C., Baltimore, November 2-6, 2010

1147/T: Frequency of 27-bp deletion mutation, another earwax determinant, in ABCC11 among the Japanese population.(1447) (5:00PM-6:00PM on Thu)

Author(s): A. Yamada, Y. Hori, Y. Ono, N. Matsuda, D. Starenki, N. Sosonkina, K. Yoshiura, T. Ohta, N. Niikawa

2219/F: Re-sequencing analysis of candidate region for a neurodegenerative disorder by massively parallel sequencing. T. Kaname, A. Tsujino, K. Yanagi, K. Hayashi, M. Tsukahara, K. Fujimori, I. Kikuzato, M. Teruya, Y. Imada, M. Nezuo, S. Yano, Y. Sato, Y. Miwa, T. Niikawa, K. Yoshiura, K. Naritomi

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

書籍

なし

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Tsuda M, Yamada T, Mikoya T, Sogabe I, Nakashima M, Minakami H, Kishino T, Kinoshita A, Niikawa N, Hirano A, Yoshiura K.	A type of familial cleft of the soft palate maps to 2p24.2-p24.1 or 2p21-p12.	***Jounal of Human Genetics***	55(2)	124-126	2010
Takahata T, Yamada K, Yamada Y, Ono S, Kinoshita A, Matsuzaka T, Yoshiura KI, Kitaoka T.	Novel mutations in the SIL1 gene in a Japanese pedigree with the Marinesco-Sjögren syndrome.	***Jounal of Human Genetics***	55(3)	142-146	2010
Miura K, Miura S, Yoshiura K, Seminara S, Hamaguchi D, Niikawa N, Masuzaki H.	A case of Kallmann syndrome carrying a missense mutation in alternatively spliced exon 8A encoding the immunoglobulin-like domain IIIb of fibroblast growth factor receptor 1.	***Human Reproduction***	25(4)	1076-1080	2010

Ng SB, Bigham AW, Buckingham KJ, Hannibal MC, McMillin MJ, Gildersleeve HI, Beck AE, Tabor HK, Cooper GM, Mefford HC, Lee C, Turner EH, Smith JD, Rieder MJ, Yoshiura K, Matsumoto N, Ohta T, Niikawa N, Nickerson DA, Bamshad MJ, Shendure J.	Exome sequencing identifies MLL2 mutations as a cause of Kabuki syndrome.	***Nature Genetics***	42(9)	790-793	2010
Matsuzawa N, Kondo S, Shimozato K, Nagao T, Nakano M, Tsuda M, Hirano A, Niikawa N, Yoshiura K.	Two missense mutations of the IRF6 gene in two Japanese families with popliteal pterygium syndrome.	***American Journal of Medical Genetics part A.***	152A(9)	2262-2267	2010
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Oikawa M, Kuniba H, Kondoh T, Kinoshita A, Nagayasu T, Niikawa N, Yoshiura K.	Familial brain arteriovenous malformation maps to 5p13-q14, 15q11-q13 or 18p11: linkage analysis with clipped fingernail DNA on high-density SNP array.	***European Journal of Medical Genetics***	53(5)	244-249	2010
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Ota I, Sakurai A, Toyoda Y, Morita S, Sasaki T, Chishima T, Yamakado M, Kawai Y, Ishidao T, Lezhava A, Yoshiura K-i, Togo S, Hayashizaki Y, Ishikawa T, Ishikawa T, Endo I and Shimada H.	Association between breast cancer risk and the wild-type allele of human ABC transporter ABCC11.	***Anticancer Research***	30(12)	5189-5194	2010

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SHORT COMMUNICATION

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

Masayoshi Tsuda[1,2,9], Takahiro Yamada[3,9], Tadashi Mikoya[4], Izumi Sogabe[5], Mitsuko Nakashima[1,2,6], Hisanori Minakami[3], Tatsuya Kishino[7], Akira Kinoshita[1], Norio Niikawa[8], Akiyoshi Hirano[2] and Koh-ichiro Yoshiura[1]

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

INTRODUCTION

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

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

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

MATERIALS AND METHODS

Family and patients

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

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

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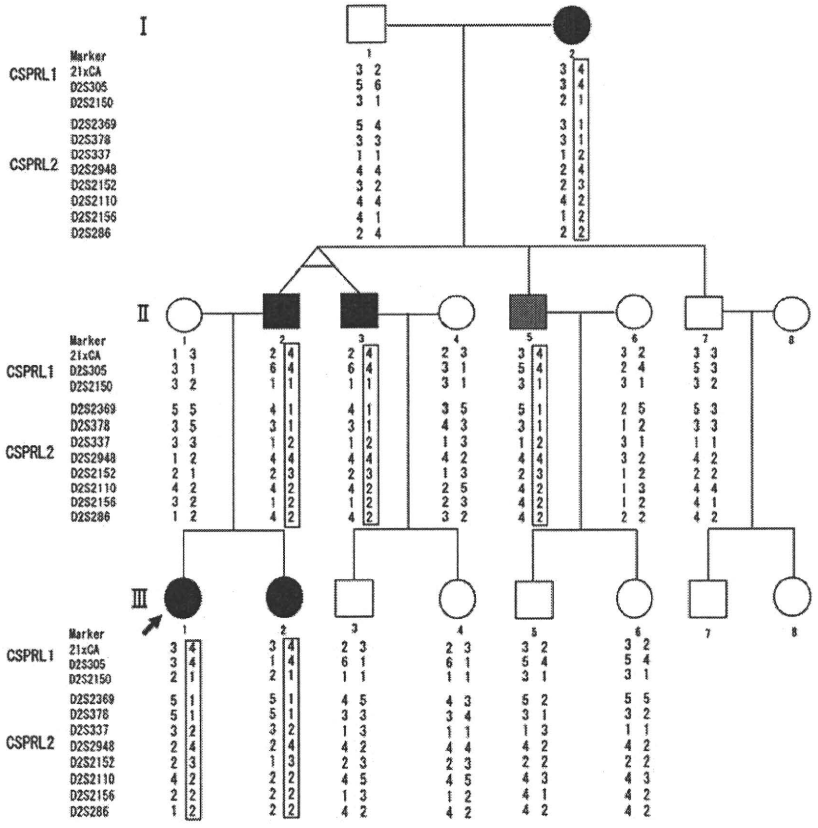

Figure 1 Family tree with haplotypes at 2p24.2–24.1 (CSPR1) and 2p21–p12 (CSPR2). Black closed, gray closed and open symbols indicate affected with cleft of the soft palate (CSP), affected with subcutaneous cleft palate (SMCP) and unaffected, respectively. An arrow indicates the proband. Genotypes of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

SNP genotyping and linkage analysis

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

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

RESULTS AND DISCUSSION

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

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

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

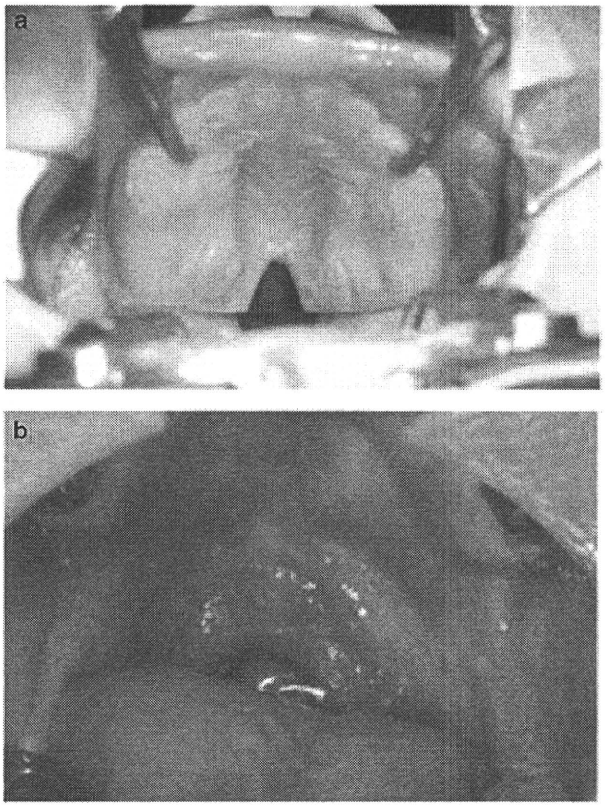

Figure 2 Views of palates. The palate of individual III-1 with CSP (**a**) showing a cleft limited to the soft palate, and that of individual II-5 with SMCP (**b**) showing a translucent zone in the soft palate resulting from a separation of the muscle.

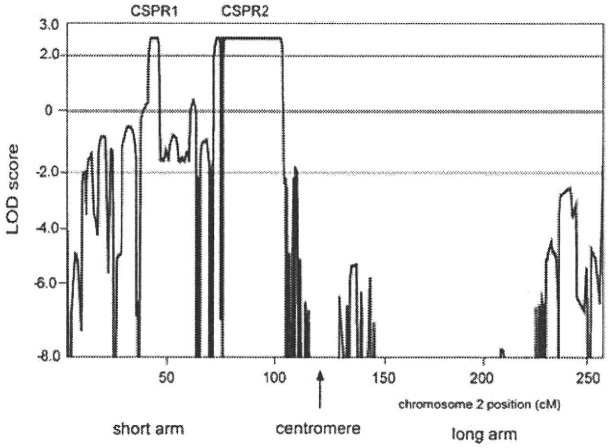

Figure 3 Multipoint LOD scores on chromosome 2. A 4.5-Mb (physical position, 18281893–22775527) interval from rs1545497 to rs1872325 corresponds to CSPR1, and a 34.5-Mb interval (45834656–80355227) from rs940053 to rs310777 corresponds to CSPR2.

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

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ORIGINAL ARTICLE

Novel mutations in the *SIL1* gene in a Japanese pedigree with the Marinesco–Sjögren syndrome

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Marinesco–Sjögren syndrome (MSS) is a rare autosomal recessive disorder. Mutation in the *SIL1* gene accounts for the majority of MSS cases. However, some individuals with typical MSS without *SIL1* mutations have been reported. In this study, we identified two novel mutations in a Japanese pedigree with MSS, one of which was an intragenic deletion not detected using the PCR-direct sequencing protocol. This family consisted of three affected siblings, an unaffected sibling and unaffected parents. We found a homozygous 5-bp deletion, del598–602(GAAGA), in exon 6 of all affected siblings by PCR. Thus, we expected that both parents would be heterozygous for the mutation. As expected, the father was heterozygous, whereas the mother demonstrated no mutations. We then carried out array comparative genomic hybridization and quantitative PCR analyses, and identified an approximately 58 kb deletion in exon 6 in the patients and mother. As a result, the mother was hemizygous for a 58-kb deletion. The affected siblings contained two mutations, a 5-bp and a 58-kb deletion, resulting in *SIL1* gene dysfunction. It is possible that some reported cases of MSS without base alterations in the *SIL1* gene are caused by deletions rather than locus heterogeneity.
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Keywords: array CGH; Marinesco–Sjögren syndrome; quantitative PCR; *SIL1* gene

INTRODUCTION

The Marinesco–Sjögren syndrome (MSS, OMIM 248800) is a rare, autosomal recessive disorder characterized by congenital cataracts, cerebellar ataxia, myopathy and mental retardation. Skeletal abnormalities including short stature, dysarthria, nystagmus and hypergonadotropic hypogonadism are also occasionally observed.

MSS was first described by Marinesco *et al.* in four Rumanian siblings in 1931.[1,2] Sjögren later reported 14 similar cases in six Swedish families and suggested an underlying autosomal recessive pattern of inheritance in 1950.[1,3] In 2003, Lagier-Tourenne *et al.*[4] identified a locus for MSS on chromosome 5q31 using homozygosity mapping in two consanguineous families of Turkish and Norwegian origin. Two years later, two groups independently identified several mutations in the *SIL1* gene located at chromosome 5q31 in MSS. In addition, Senderek *et al.*[5] identified nine different mutations in eight MSS families, and Anttonen *et al.*[6] found four different mutations in eight MSS families. Further novel mutations in the *SIL1* gene in MSS were subsequently identified.[7–9] Although mutations in the *SIL1* gene account for the majority of MSS cases, Senderrek *et al.*[5] reported four individuals with typical MSS lacking *SIL1* mutations. These reports suggest genetic heterogeneity in MSS. Here, we report novel mutations, including a deletion that is difficult to detect using conventional PCR for sequence analysis, in the *SIL1* gene in a Japanese family that included three individuals with MSS.

MATERIALS AND METHODS

Family

A Japanese family with MSS was investigated in this study. The clinical features of the affected individuals are summarized in Table 1. Each individual was diagnosed on the basis of the clinical features of MSS. The family consisted of three affected siblings, an unaffected sibling and their parents (Figure 1a). Parents were not consanguineous and all affected individuals were born after normal pregnancies.

The ophthalmological clinical features were as follows: The proband (II-1 in Figure 1a), an affected 14-year-old daughter, demonstrated slight bilateral cataract at 3 years of age, and received an operation for cataract at 4 years of age. Her best-corrected visual acuity after the operation was 1.0. An affected 10-year-old son (II-3) demonstrated slight bilateral posterior subcapsular cataracts at age 1 year and 6 months, and underwent an operation for cataract at 3 years of age. His best-corrected visual acuity after the operation was 1.2. An affected 8-year-old daughter (II-4) demonstrated bilateral total cataract at 4

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Table 1 Clinical features of the affected individuals

	II-1	*II-3*	*II-4*
Sex	F	M	F
Age	14	10	8
Bilateral cataract	+	+	+
Nystagmus	–	–	–
Strabismus	+	+	+
Skeletal deformities	+	+	+
Ataxia	+	+	+
Hypotonia	+	+	+
Spasticity	+	+	+
Mental retardation	+	+	+
Elevated serum CK	+	+	+
Myopathic EMG	+	NA	NA
Myopathic biopsy	+	NA	NA

Abbreviations: –, absent; +, present; CK, creatine kinase; EMG, electromyography; F, female; M, male; NA, not available.

years of age, and received an operation for cataract at the same age. Her best-corrected visual acuity after the operation was 0.6. Further ophthalmological examination of the three affected children revealed no abnormalities.

All samples from the family were collected after obtaining written informed consent, and the study protocol was preapproved by the Committee for the Ethical Issues on Human Genome and Gene Analysis in Nagasaki University. Genomic DNA was extracted directly from blood using the QIAamp DNA Blood mini kit (Qiagen, Hilden, Germany).

Mutation analysis

To identify mutations in the *SIL1* gene, PCR products were subjected to the direct sequencing protocol. Information regarding primer sequences was kindly provided by Dr Senderek (Department of Human Genetics, Aachen University of Technology) and Dr Anttonen (Folkhälsan Institute of Genetics and Neuroscience Center and Department of Medical Genetics, University of Helsinki). PCR was performed in a 30-μl reaction mixture containing 30 ng genomic DNA, 0.5 μM each of forward and reverse primers, 200 μM each of dNTP in 1× ExTaq buffer (Takara Bio, Shiga, Japan) and 0.75 U ExTaq (Takara Bio). PCR was performed in an iCycler thermal cycler (Bio-Rad, Hercules, CA, USA) and the PCR conditions were as follows: Taq activation step at 95 °C for 4 min, followed by 35 cycles at 95 °C for 30 s for denaturation, 58 °C for 30 s for annealing, 72 °C for 30 s for extension and finally one step at 72 °C for 10 min to ensure complete extension.

The PCR products were treated with ExoSAP-IT (USB, Cleveland, OH, USA) and directly sequenced using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). Samples were run on an ABI 3130-xl automated sequencer (Applied Biosystems) and electropherograms were aligned using ATGC software version 5.0 (Genetyx, Tokyo, Japan). Mutations were inspected visually.

Microsatellite analysis

Microsatellite analyses were carried out using the ABI PRISM linkage Mapping Set-MD10 (panel 8) and included eight markers on chromosome 5. PCR was performed in a 15-μl reaction mixture under the same conditions for the mutation analysis, with the exception that 55 °C for 30 s was used for annealing.

After mixing with GeneScan 400HD ROX size standard (Applied Biosystems) in deionized formamide, amplicons were separated on the ABI 3130-xl automated sequencer. Genotyping data were analyzed using GeneMapper 4.0 software (Applied Biosystems).

Array CGH

A DNA sample from patient II-3 (Figure 1a) was subjected to the high-density oligonucleotide-based array comparative genomic hybridization (CGH) assay. For this assay, we manufactured a custom-designed microarray targeted to a 300-kb genome region, including the *SIL1* gene, on 5q31.2 (Chr5:138 281 500–138 580 000 [NCBI Build 36.1, hg18]). We used the Agilent website (http://earray.chem.agilent.com/earray/) to design our custom array CGH. This array contained 2685 probes that were 60-mer in length (Agilent Technologies, Santa Clara, CA, USA). Experiments were performed according to the manufacturer's instructions. Briefly, patient and reference genomic DNA samples (1 μg per sample) were fluorescently labeled with Cy3 (patient) and Cy5 (reference) using the Agilent Genomic DNA Labeling Kit (Agilent Technologies). Labeled patient and reference DNA was then combined, denatured and preannealed with Cot-1 DNA (Invitrogen, Carlsbad, USA, USA) and blocking reagent (Agilent Technologies). The labeled samples were then hybridized to the arrays for 40 h in a rotating oven (Agilent Technologies) at 65 °C and 20 r.p.m. After hybridization and washing, the arrays were scanned at a 5-μm resolution with an Agilent G2565C scanner. The resulting images were analyzed using Feature Extraction Software 10.5.1.1 (Agilent Technologies).

Quantitative PCR analysis

Real-time PCR was performed using a LightCycler 480 Instrument (Roche Applied Science, Penzberg, Germany) and SYTO13 dye (Molecular Probes, Eugene, OR, USA). Exons 2, 6 and 10 of the *SIL1* gene were selected as target exons for quantification. The *NSD1* gene was used as a reference gene (two copies in the reference DNA). Primers for the *SIL1* and *NSD1* genes were designed using Primer Express 1.5 (Applied Biosystems) and are listed in Table 2. Real-time PCR was performed in a 10-μl reaction mixture containing 10 ng genomic DNA, 0.5 μM each of forward and reverse primers, 200 μM each of dNTP, 1× ExTaq buffer, 0.2 μM SYTO13 dye and 0.5 U ExTaq.

Break-point determination

To determine the break point of deletion, we designed a deletion-specific amplification primer around the deletion break point detected by array CGH. The primer sequences were 5′-AGCGGATCAGTAAGGGTATT-3′ for SILint5-delF and 5′-CAGTGTCTGGAAGCACAAGC-3′ for SILint7delR. DNA from all family members and from 80 healthy individuals was subjected to PCR amplification using the same conditions as the mutation analysis, with the exception that 61 °C was used as the annealing temperature.

RESULTS

Mutation analysis

We sequenced all 10 exons of the *SIL1* gene in our MSS family members. Portions of the electropherograms are presented in Figure 1b. The electropherograms for the three affected siblings demonstrated that they were homozygous for a 5-bp deletion mutation, del598-602(GAAGA), in exon 6. No mutations were identified in the unaffected sibling. A heterozygous del598-602(GAAGA) mutation in exon 6 was also detected in the father. The mother's electropherogram did not show del598-602(GAAGA). del598-602(GAAGA) was not detected in any of the 80 healthy Japanese individuals.

Microsatellite analysis

All the eight microsatellite markers investigated showed the concordant inheritance pattern of the allele from both parents. This means that the parent–child relationship was confirmed, and that long-range uniparental disomy could be excluded. The inheritance pattern of the allele on chromosome 5 is summarized in Figure 1a.

Copy number analysis in the family members

We speculated that the patients and mother had a deletion in exon 6 of the *SIL1* gene on the basis of microsatellite and mutation analyses. We were able to identify the deletion within the *SIL1* genomic region using real-time PCR and array CGH (Figures 2 and 3a). The copy number state in the patients determined by real-time PCR was concordant with the results of array CGH (Figure 2).

Deletion break point

Using SILint5delF/SILint7delR primers we were able to amplify PCR products of the patients and mother, but not of the healthy

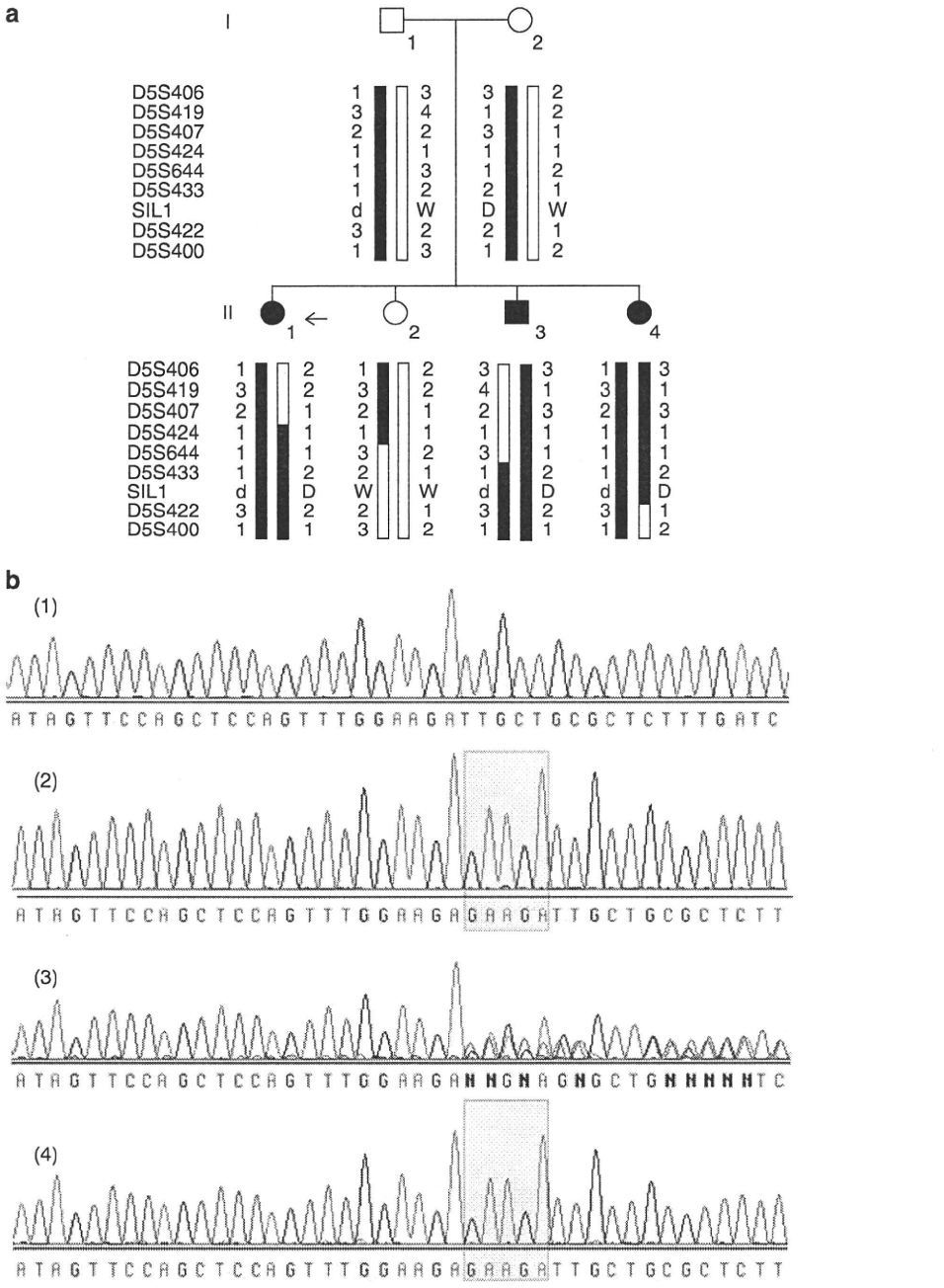

Figure 1 Microsatellite and mutation analysis. (**a**) Pedigree of the MSS family studied and the marker haplotypes. Closed symbols indicate individuals with MSS and open symbols represent clinically unaffected individuals. All investigated allele sizes of the four siblings corresponded to the allele sizes of either parent. The locus for the *SIL1* gene is between D5S433 and D45422, d: 5 bp deletion in exon 6, D: deletion including exon 6, W: wild type. Arrow indicates proband. (**b**) DNA sequence data in our MSS family. (1) Affected sibling (II-4 in panel a) with the homozygous del598-602(GAAGA) mutation, (2) unaffected sibling (II-2) without the mutation, (3) father (I-1) with the heterozygous del598-602(GAAGA) mutation and (4) mother without a mutation.

individuals. The deletion-specific product was subsequently processed for sequence analysis, confirming the 58 269-bp deletion [ch5:g.(138 339 032–138 397 300)del](NCBI Build 36.1, hg18) and the 4-bp insertion (Figure 3b). The telomeric break point within intron 5 was in the LINE/L1 repetitive sequence, whereas the centromeric break point within intron 7 was a unique sequence.

DISCUSSION

In this study, we identified novel mutations in the *SIL1* gene in a Japanese family that included three children with MSS. We sequenced all 10 exons of the *SIL1* gene, and identified a del598-602(GAAGA) mutation in exon 6 of the PCR products amplified from genomic DNA isolated from all three of the affected siblings. Thus, we expected