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
Miura K, Miura S, Yamasaki K, Shimada T, Kinoshita A, Niikawa N, Yoshiura K, Masuzaki H.	The possibility of microarray-based analysis using cell-free placental mRNA in maternal plasma.	***Prenatal Diagnosis***	30(9)	849-861	2010
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
Ono S, Imamura A, Tasaki S, Kurotaki N, Ozawa H, Yoshiura K, Okazaki Y.	Failure to Confirm CNVs as of Aetiological Significance in Twin Pairs Discordant for Schizophrenia.	***Twin Research and Human Genetics***	13(5)	455-460	2010
Miura K, Miura S, Yamasaki K, Higashijima A, Kinoshita A, Yoshiura KI, Masuzaki H.	Identification of Pregnancy-Associated MicroRNAs in Maternal Plasma.	***Clinical Chemistry***	56(1)	1767-1771	2010

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

平成２３年度研究成果の刊行に関する一覧表

書籍

平成２３年度の書籍刊行物なし

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
Okada I, Hamanoue H., Terada K, Tohma T, Megarbane A, Chouery E, Abou-Ghoch J, Jalkh N, Cogulu O, Ozkinay F, Horie K, Takeda J, Furuichi T, Ikegawa S, Nishiyama K, Miyatake S, Nishimura A, Mizuguchi T, Niikawa N, Hirahara F, Kaname T, Yoshiura K, Tsurusaki Y, Doi H, Noriko Miyake N, Furukawa T, Matsumoto N, and Saitsu H.	SMOC1 Is Essential for Ocular and Limb Development in Humans and Mice.	***American Journal of Human Genetics***	88(1)	1-12	2011
Amani D, Ravangard F, Niikawa N, Yoshiura KI, Karimzadeh M, Dehaghani AS, Ghaderi A.	Coding region polymorphisms in the indoleamine 2,3-dioxygenase (INDO) gene and recurrent spontaneous abortion.	***Jounal of Reproductive Immunology***	88(1)	42-47	2011
Ono S, Yoshiura K, Kurotaki N, Kikuchi T, Niikawa N, Kinoshita A.	Mutation and Copy Number Analysis in Paroxysmal Kinesigenic Dyskinesia Families.	***Movement Disorders***	26(4)	762-764	2011
Miura K, Higashijima A, Shimada T, Miura S, Yamasaki K, Abe S, Jo O, Kinoshita A, Yoshida A, Yoshimura S, Niikawa N, Yoshiura K, Masuzaki H.	Clinical application of fetal sex determination using cell-free fetal DNA in pregnant carriers of X-linked genetic disorders.	***Journal of Human Genetics***	56(4)	296-299	2011

Yamasaki K, Miura K, Shimada T, Miura S, Abe S, Murakami M, Sameshima T, Fujishita A, Kotera K, Kinoshita A, Yoshiura K, Masuzaki H.	Epidemiology of human papillomavirus genotypes in pregnant Japanese women.	***Journal of Human Genetics***	56(4)	313-315	2011
Kurotaki N, Tasaki S, Mishima H, Ono S, Imamura A, Kikuchi T, Nishida N, Tokunaga K, Yoshiura K, Hiroki Ozawa H.	Identification of Novel Schizophrenia Loci by Homozygosity Mapping Using DNA Microarray Analysis.	***PLoS One***	6(5)	e20589	2011
Oikawa M, Nagayasu T, Yano H, Hayashi T, Abe K, Kinoshita A, Yoshiura KI.	Intracystic Papillary Carcinoma of Breast Harbors Significant Genomic Alteration Compared with Intracystic Papilloma: Genome-wide Copy Number and LOH Analysis Using High-Density Single-Nucleotide Polymorphism Microarrays.	***Breast Journal***	17(4)	427-430	2011
Mishima H, Sasaki K, Tanaka M, Tatebe O, Yoshiura KI.	Agile parallel bioinformatics workflow management using Pwrake.	***BMC Res Notes***	4(1)	331	2011
Hannibal MC, Buckingham KJ, Ng SB, Ming JE, Beck AE, McMillin MJ, Gildersleeve HI, Bigham AW, Tabor HK, Mefford HC, Cook J, Yoshiura K, Matsumoto T, Matsumoto N, Miyake N, Tonoki H, Naritomi K, Kaname T, Nagai T, Ohashi H, Kurosawa K, Hou JW, Ohta T, Liang D, Sudo A, Morris CA, Banka S, Black GC, Clayton-Smith J, Nickerson DA, Zackai EH, Shaikh TH, Donnai D, Niikawa N, Shendure J, Bamshad MJ.	Spectrum of MLL2 (ALR) mutations in 110 cases of Kabuki syndrome.	***American Journal of Medical Genetics, Part A***	155A(7)	1511-1516	2011

Arima K, Kinoshita A, Mishima H, Kanazawa N, KanekoT, Mizushima T, Ichinose K, Nakamura H, Tsujino A, Kawakami A, Matsunaka M, Kasagi S, Kawano S, Kumagai S, Ohmura K, Mimori T, Hirano M, Ueno S, Tanaka K, Tanaka M, Toyoshima I, Sugino H, Yamakawa A, Tanaka K, Niikawa N, Furukawa F, Shigeo Murata S, Eguchi K, Ida H, Yoshiura K.	An assembly defect due to a *PSMB8* mutation reduces proteasome activity and causes autoinflammatory disorder, Nakajo-Nishimura syndrome.	***Procedings National Academy of Science,* USA**	108(36)	14914-14919	2011
Yamasaki K, Miura K, Shimada T, Ikemoto R, Miura S, Murakami M, Sameshima T, Fujishita A, Kotera K, Kinoshita A, Yoshiura KI, Masuzaki H.	Pre-vaccination epidemiology of human papillomavirus infections in Japanese women with abnormal cytology.	***Jouna; of Obstetrics and Gynaecological Research***	37(11)	1666-1670.	2011
Ono S, Tanaka T, Ishida M, Kinoshita A, Fukuoka J, Takaki M, Sakamoto N, Ishimatsu Y, Kohno S, Hayashi T, Senba M, Yasunami M, Kubo Y, Yoshida LM, Kubo H, Ariyoshi K, Yoshiura K, Morimoto K.	Surfactant protein C G100S mutation causes familial pulmonary fibrosis in Japanese kindred.	***European Respiratory Journal***	38(4)	861-869	2011
Sasaki K, Okamoto N, Kosaki K, Yorifuji T, Shimokawa O, Mishima H, Yoshiura K-i, Harada N.	Maternal uniparental isodisomy and heterodisomy on chromosome 6 encompassing a *CUL7* gene mutation causing 3M syndrome.	***Clinical Genetics***	80(5)	478-483	2011
Oikawa M, Yoshiura KI, Kondo H, Miura S, Nagayasu T, Nakashima M.	Significance of Genomic Instability in Breast Cancer in Atomic Bomb Survivors: Analysis of Microarray-Comparative Genomic Hybridization.	***Radiation Oncology***	6(1)	168	2011

Hikida M, Tsuda M, Watanabe A, Kinoshita A, Akita S, Hirano A, Uchiyama T, Yoshiura KI.	No evidence of association between 8q24 and susceptibility to nonsyndromic cleft lip with or without palate in Japanese population.	***Cleft Palate Craniofacial Journal***	In press		2011
Tsurusaki Y, Okamoto N, Ohashi H, Kosho T, Imai Y, Hibi-Ko Y, Kaname T, Naritomi K, Kawame H, Wakui K, Fukushima Y, Homma T, Kato M, Hiraki Y, Yamagata T, Yano S, Mizuno S, Sakazume S, Ishii T, Nagai T, Shiina M, Ogata K, Ohta T, Niikawa N, Miyatake S, Okada I, Mizuguchi T, Doi H, Saitsu H, Miyake N, Matsumoto N.	Mutations affecting components of the SWI/SNF complex cause Coffin-Siris syndrome.	***Nature Genetics***	44(4)	376-378	2011

III. 研究成果の刊行物・別冊

Journal of Human Genetics (2010) 55, 124–126

www.nature.com/jhg

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.
Journal of Human Genetics (2010) **55**, 124–126; doi:10.1038/jhg.2009.131; published online 15 January 2010

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.

[1]Department of Human Genetics, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan; [2]Department of Plastic and Reconstructive Surgery, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan; [3]Department of Obstetrics and Gynecology, Hokkaido University Graduate School of Medicine, Sapporo, Japan; [4]Center for Advanced Oral Medicine, Hokkaido University Hospital, Sapporo, Japan; [5]Department of Maxillofacial Surgery, Hokkaido University Graduate School of Medicine, Sapporo, Japan; [6]Laboratory of Molecular Medicine and Laboratory of Genome Technology of the Human Genome Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan; [7]Division of Functional Genomics, Center for Frontier Life Sciences, Nagasaki University, Nagasaki, Japan and [8]Research Institute of Personalized Health Sciences, Health Sciences University of Hokkaido, Tobetsu, Japan
[9]These authors contributed equally to this work.
Correspondence: Dr K-i Yoshiura, Department of Human Genetics, Nagasaki University, Graduate School of Biomedical Sciences, 1-12-4, Sakamoto, Nagasaki 852-8523, Japan.
E-mail: kyoshi@nagasaki-u.ac.jp
Received 26 July 2009; revised 30 October 2009; accepted 13 November 2009; published online 15 January 2010

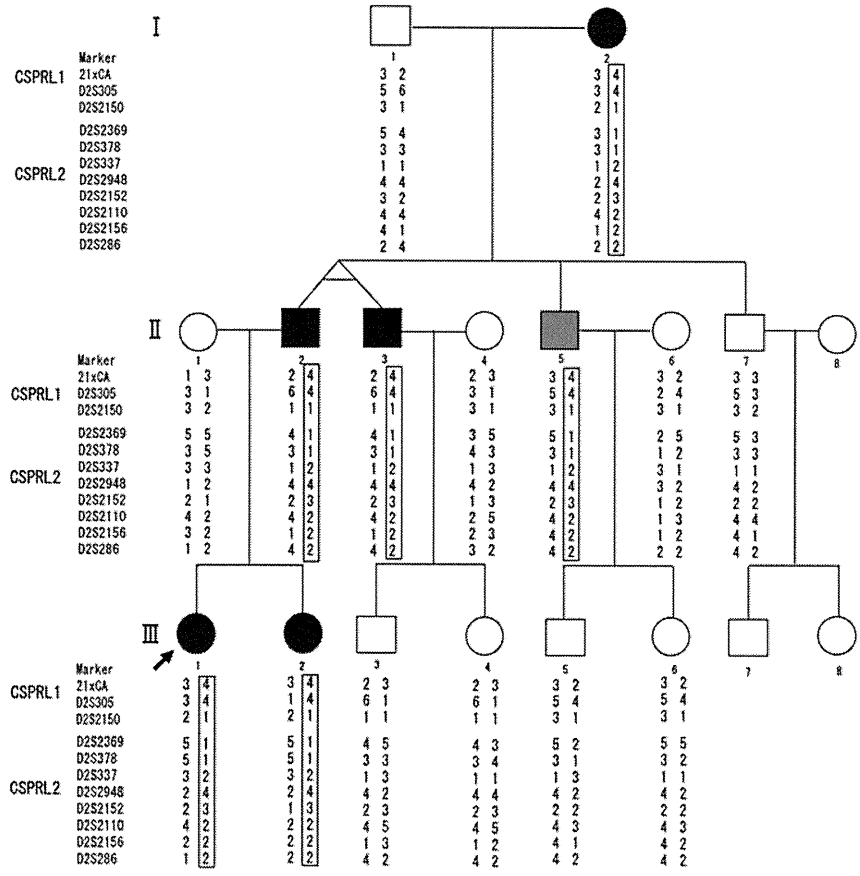

Figure 1 Family tree with haplotypes at 2p24.2–24.1 (CSPR1) and 2p21–p12 (CSPR2). Black closed, gray closed and open symbols indicate affected with cleft of the soft palate (CSP), affected with subcutaneous cleft palate (SMCP) and unaffected, respectively. An arrow indicates the proband. Genotypes of 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.

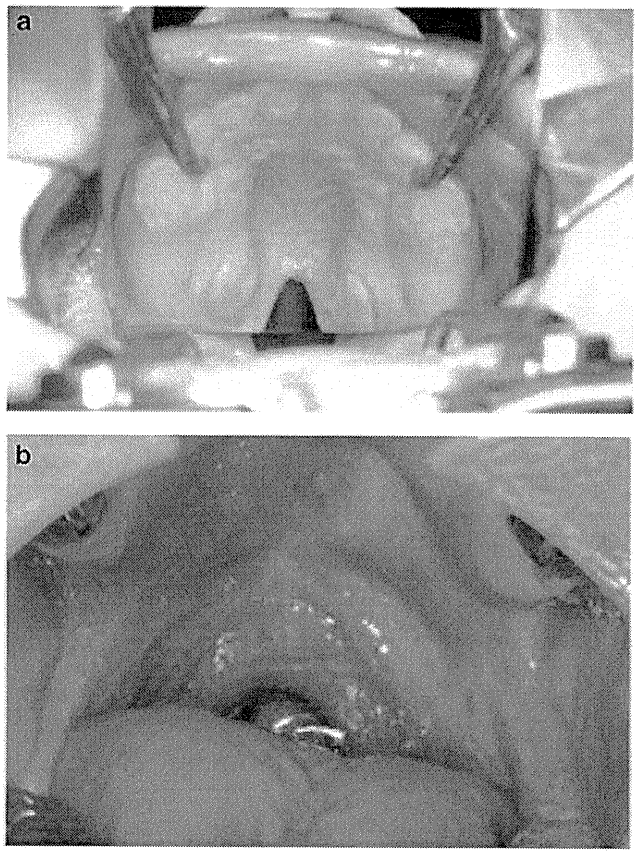

Figure 2 Views of palates. The palate of individual III-1 with CSP (**a**) showing a cleft limited to the soft palate, and that of individual II-5 with SMCP (**b**) showing a translucent zone in the soft palate resulting from a separation of the muscle.

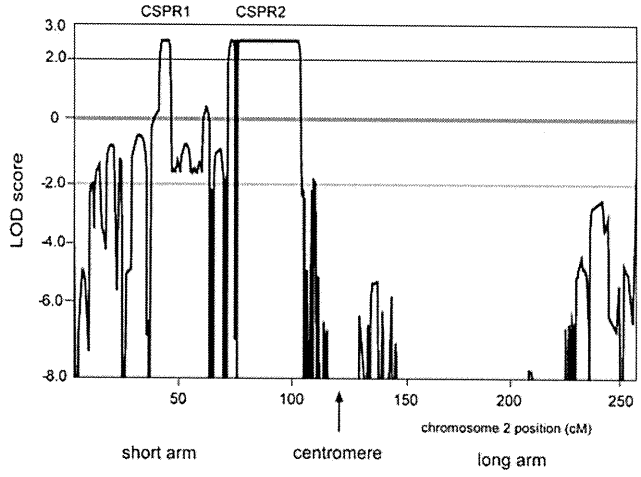

Figure 3 Multipoint LOD scores on chromosome 2. A 4.5-Mb (physical position, 18281893–22775527) interval from rs1545497 to rs1872325 corresponds to CSPR1, and a 34.5-Mb interval (45834656–80355227) from rs940053 to rs310777 corresponds to CSPR2.

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.

ACKNOWLEDGEMENTS

We are grateful to the members of the family for their participation in this research. We also thank Ms Miho Ooga and Ms Chisa Hayashida for their technical assistance. KY was supported partly by a Grant-in-Aid for Scientific Research from the Ministry of Health, Labour and Welfare, and partly by grants from the Takeda Scientific Foundation and the Naito Foundation.

1 FitzPatrick, D. R., Carr, I. M., McLaren, L., Leek, J. P., Wightman, P., Williamson, K. *et al.* Identification of SATB2 as the cleft palate gene on 2q32-q33. *Hum. Mol. Genet.* **12,** 2491–2501 (2003).
2 Braybrook, C., Doudney, K., Marçano, A. C., Arnason, A., Bjornsson, A., Patton, M. A. *et al.* The T-box transcription factor gene TBX22 is mutated in X-linked cleft palate and ankyloglossia. *Nat. Genet.* **29,** 179–183 (2001).
3 Koillinen, H., Lahermo, P., Rautio, J., Hukki, J., Peyrard-Janvid, M. & Kere, J. A. Genome-wide scan of non-syndromic cleft palate only (CPO) in Finnish multiplex families. *J. Med. Genet.* **42,** 177–184 (2005).
4 Shiang, R., Lidral, A. C., Ardinger, H. H., Buetow, K. H., Romitti, P. A. & Munger, R. G. Association of transforming growth-factor alpha gene polymorphisms with nonsyndromic cleft palate only (CPO). *Am. J. Hum. Genet.* **53,** 836–843 (1993).
5 Hwang, S. J., Beaty, T. H., Panny, S. R., Street, N. A., Joseph, J. M., Gordon, S. *et al.* Association study of transforming growth factor alpha (TGF alpha) TaqI polymorphism and oral clefts: indication of gene-environment interaction in a population-based sample of infants with birth defects. *Am. J. Epidemiol.* **141,** 629–636 (1995).
6 Carinci, F., Scapoli, L., Palmieri, A., Zollino, I. & Pezzetti, F. Human genetic factors in nonsyndromic cleft lip and palate: An update. *Int. J. Pediatr. Otorhinolaryngol.* **71,** 1509–1519 (2007).
7 Millard, D. R. in *Cleft Craft, the Evolution of its Surgery, Vol 1 The Unilateral Deformity* (eds Millard, D.R.) 41–55 (Little Brown, Boston, 1976).
8 Calnan, J. Submucous cleft palate. *Br. J. Plast. Surg.* **6,** 264–282 (1954).
9 Christensen, K. & Fogh-Anderson, P. Etiological subgroups in non-syndromic isolated cleft palate. A genetic-epidemiological study of 52 Danish birth cohorts. *Clin. Genet.* **46,** 329–335 (1994).
10 Abecasis, G. R., Cherny, S. S., Cookson, W. O. & Cardon, L. R. Merlin–rapid analysis of dense genetic maps using sparse gene flow trees. *Nat. Genet.* **30,** 97–101 (2002).
11 O'Connell, J. R. & Weeks, D. E. PedCheck: a program for identification of genotype incompatibilities in linkage analysis. *Am. J. Hum. Genet.* **63,** 259–266 (1998).
12 Nakashima, M., Nakano, M., Hirano, A., Kishino, T., Kondoh, S., Miwa, N. *et al.* Genome-wide linkage analysis and mutation analysis of hereditary congenital blepharoptosis in a Japanese family. *J. Hum. Genet.* **53,** 34–41 (2008).
13 Lathrop, G. M., Lalouel, J. M., Julier, C. & Ott, J. Strategies for multilocus linkage analysis in humans. *Proc. Natl Acad. Sci. USA* **81,** 3443–3446 (1984).

Journal of Human Genetics (2010) 55, 142–146

www.nature.com/jhg

ORIGINAL ARTICLE

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

Taichi Takahata[1,7], Koki Yamada[1,2,7], Yoshihisa Yamada[1], Shinji Ono[3,4], Akira Kinoshita[4], Tetsuo Matsuzaka[5,6], Koh-ichiro Yoshiura[4] and Takashi Kitaoka[1]

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.
Journal of Human Genetics (2010) **55**, 142–146; doi:10.1038/jhg.2009.141; published online 29 January 2010

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

[1]Department of Ophthalmology and Visual Sciences, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan; [2]Department of Ophthalmology, Sasebo City General Hospital, Sasebo, Japan; [3]Department of Psychiatry, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan; [4]Department of Human Genetics, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan; [5]Department of Pediatrics, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan and [6]Department of Pediatrics, Nagasaki Children's Medical and Welfare Center, Isahaya, Japan
[7]These authors contributed equally to this work.
Correspondence: Dr T Takahata, Department of Ophthalmology and Visual Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan.
E-mail: takahata@nagasaki-u.ac.jp
Received 3 August 2009; revised 15 December 2009; accepted 16 December 2009; published online 29 January 2010

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

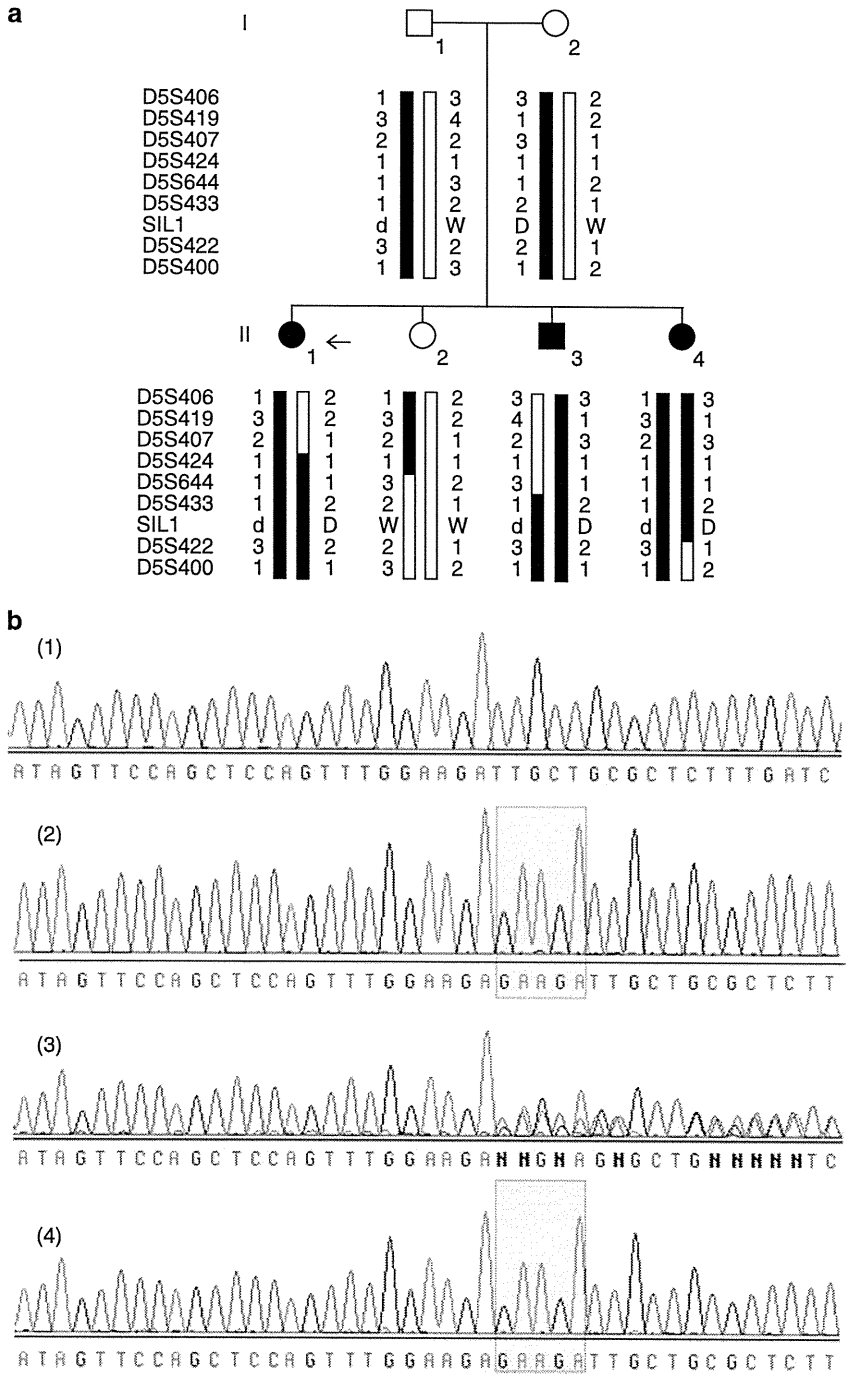

Figure 1 Microsatellite and mutation analysis. (**a**) Pedigree of the MSS family studied and the marker haplotypes. Closed symbols indicate individuals with MSS and open symbols represent clinically unaffected individuals. All investigated allele sizes of the four siblings corresponded to the allele 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

that the affected siblings would be homozygous for the mutation obtained from a parent, and that both parents would be heterozygous for the mutation. However, we found that only the father expressed the del598-602(GAAGA) mutation, whereas no mutations were identified in any of the 10 exons of the *SIL1* gene in the mother.

We next confirmed the parent–child relationship for each sibling using microsatellite markers on chromosome 5. The mutation and microsatellite analyses suggested that the mother may be hemizygous around exon 6. Quantitative PCR analyses in all family members indicated that the unaffected sibling and father expressed two copies of exon 6 in the *SLI1* gene, whereas the three affected siblings and mother expressed only one copy of exon 6. Therefore, we attempted to define the copy number state for the entire *SIL1* gene using array CGH to confirm the break point of deletion. As it is possible to speculate break points from the array CGH results, we were able to design primers to amplify the deletion-specific product using PCR. Using this method, we found a 58 269-bp deletion in the three affected siblings and mother. The character of break points was not specific, and did not indicate the recombination between the repetitive sequence or low copy repeats.

Table 2 Primer sequences for real-time PCR

Target sequence (*SIL1* exon 2)	
Forward primer	5′-CTCTTGTTGGATGGCTGGAC-3′
Reverse primer	5′-TGTGATTCCCATGTCGTCAC-3′
Target sequence (*SIL1* exon 6)	
Forward primer	5′-GGCAGATGTCTCCAACCAAT-3′
Reverse primer	5′-CTTGTTGATCAGCCGTACCA-3′
Target sequence (*SIL1* exon 10)	
Forward primer	5′-AGAGCTAGCCAGGTGTGAGC-3′
Reverse primer	5′-AGGAGGTGTACCTGGCGATA-3′
Reference sequence (*NSD1*)	
Forward primer	5′-ATGCTTTTTCAGCCCAAATG-3′
Reverse primer	5′-CTCCCTGCAGTACAGCATCA-3′

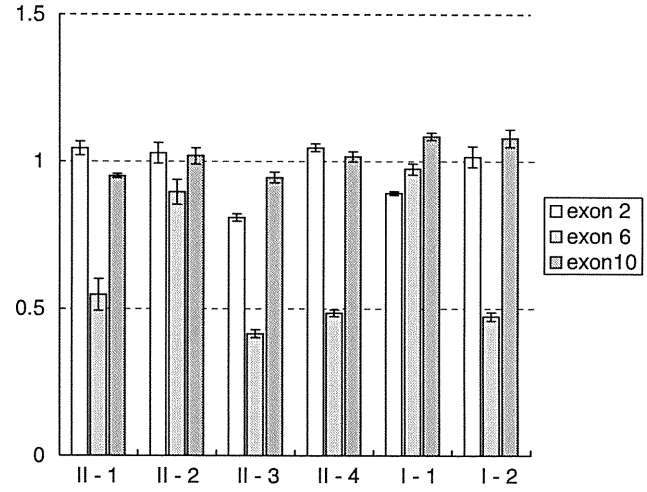

Figure 2 Copy number analysis. The *SIL1* to *NSD1* copy number ratio (*N*) for all family members. The sample with the deletion in *SIL1* is expected to yield *N*=0.5. The *N* values of exon 6 in affected siblings and the mother were 0.5472 (II-1), 0.414 (II-3), 0.483 (II-4) and 0.472 (I-1), respectively. The unaffected sibling was 0.897 (II-2) and the father (II-1) was 0.974. The fact that the *N* value of exons 2 and 10 for all family members was approximately 1.0 suggested that the deletion was not large enough to include the entire *SIL1* gene. The results are presented as mean ± s.d.

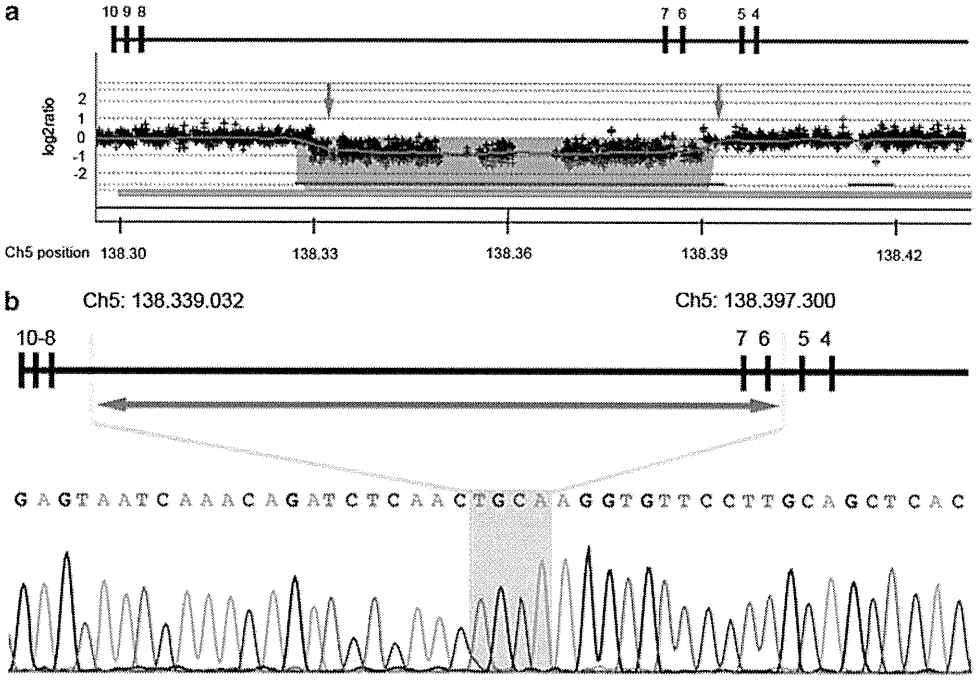

Figure 3 Break-point determination. (**a**) DNA Analytics view of the affected sibling (II-3) using the Agilent custom-designed array showing the approximately 58 kb deletion in the *SIL1* gene. Arrows indicate the break point. (**b**) The sequence results around the break point and the schematic drawing of the *SIL1* gene in the affected sibling (II-3) are shown. A 58 269 bp deletion at chr5: 138 339 032–138 397 300 (NCBI Build 36.1, hg18) and a 4b insertion (shaded region) were identified.

Table 3 Previously reported mutations in the *SIL1* gene of MSS patients

	Type	*Location*	*Nucleotide change*	*Amino-acid change*	*Origin*	P	*Ref.*
1	HM	Exon 3	212dupA	H71Qfs	France	1	6
2	HM	Exon 4	331C>T	R111X	Iran, Turkey, Italy	4	5,6,10
3	HM	Exon 6	506_509dupAAGA	D170fs	Finland, Norway	5	6
4	HM	Exon 6	645+1G>A	Skipping	Turkey	1	5
5	HM	Exon 9	936dupG	L313fs	Japan	2	8,9
6	HM	Exon 9	1029+1G>A	Skipping	Bosnia	1	5
7	HM	Exon 9	1030-9G>A	F345fs	Norway	2	9
8	HM	Exon 9	1249C>T	Q417X	Mali	1	5
9	HM	Exon 10	1312C>T	Q438X	Egypt	1	7
10	HM	Exon 10	1367T>A	L456X	Turkey	1	9
11	HM	Exon 10	1370T>C	L457Pro	Japan	1	9
12	CH	Exons 2, 4	178G>T 346delG	E60X G116fs	Vietnam	1	5
13	CH	Exon 6	506_509dupAAGA 645+2T>C	D170fs skipping	Sweden	1	6
14	CH	Exons 9, 10	947_948insT 1030-18G>A	L316fs M344fs	Germany	1	5
15	CH	Exons 9, 10	947_948insT 1366delT	L316fs 456fs	Russia	1	5

Abbreviations: CH, compound heterozygous; del, deletion; dup, duplication; fs, frameshift; HM, homozygous; ins, insertion; MSS, Marinesco–Sjögren syndrome; P, pedigree number; Ref., reference; X, stop.

MSS is a rare, autosomal recessive disorder. After the two initial groups independently identified several mutations in the *SIL1* gene in 2005,[5,6] only a few mutations in the *SIL1* gene have been reported since.[7–10] Karim *et al.*[7] located a novel mutation in an Egyptian family in 2006, and Eriguchi *et al.*[8] identified a novel mutation in three unrelated Japanese patients in 2008. All mutations in the *SIL1* gene reported previously to be associated with MSS are presented in Table 3. The mutation we found was located in exon 6, which encodes the BiP-interacting domain.[5] Zhao *et al.*[11] have reported that the SIL1 protein associates with the BiP chaperone to aid unfolded proteins in folding normally, and to help in the release of folded proteins. Thus, the loss of SIL1 protein function results in BiP recycling and the accumulation of unfolded proteins in the endoplasmic reticulum.[11–13]

Senderek *et al.*[5] were unable to identify any *SIL1* gene mutations in four individuals with typical MSS. These reports suggested genetic heterogeneity in MSS or that individuals exhibiting MSS may contain mutations that are difficult to detect. For example, compound heterozygous deletions that include different exons or intronic base changes affect the splicing process. In general, when gene mutations in a single gene defect syndrome are detected, it is essential to consider that deletion may not be detected using the PCR-direct sequencing protocol. Our results suggested that deletion assay, quantitative PCR, array CGH or multiple ligation-mediated PCR amplification should be performed to detect deletions of exons in MSS patients. It remains possible that some reported cases without base alterations in the *SIL1* gene are caused by small deletions rather than locus heterogeneity.

ACKNOWLEDGEMENTS

K Yamada was supported partly by a Grants-in-Aid for Scientific Research Category, no. 18791284 from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT). K Yoshiura was supported partly by a Grant-in-Aid for Scientific Research from the Ministry of Health, Labour and Welfare, and partly by grants from the Takeda Scientific Foundation and the Naito Foundation. We are greatly indebted to all the participants of this research. We also thank Ms M Ooga and C Hayashida for their excellent technical assistance.

1 Andersen, B. Marinesco-Sjogren syndrome: spinocerebellar ataxia, congenital cataract, somatic and mental retardation. *Dev Med Child Neurol.* **47,** 249–257 (1965).
2 Marinesco, G., Draganesco, S. & Vasiliu, D. Novelle maladie familiale caracterisee par Une cataracte congenitale et un arret du development somato-neuro-phychique. *Lencephale.* **26,** 97–109 (1931).
3 Sjögren, T. Hereditary congenital spinocerebellar ataxia accompanied by congenital cataracts and oligophrenia. *Confin. Neurol.* **10,** 293–308 (1950).
4 Lagier-Tourenne, C., Tranebjaerg, L., Chaigne, D., Gribaa, M., Dollfus, H., Silvestri, G. *et al.* Homozygosity mapping of Marinesco-Sjogren syndrome to 5q31. *Eur. J. Hum. Genet.* **11,** 770–778 (2003).
5 Senderek, J., Krieger, M., Stendel, C., North, K., Muntoni, F., Quijano-Roy, S. *et al.* Mutations in SIL1 cause Marinesco-Sjogren syndrome, a cerebellar ataxia with cataract and myopathy. *Nat. Genet.* **37,** 1312–1314 (2005).
6 Anttonen, A. K., Mahjneh, I., Hämäläinen, R. H., Lagier-Tourenne, C., Kopra, O., Waris, L. *et al.* The gene disrupted in Marinesco-Sjogren syndrome encodes SIL1, an HSPA5 cochaperone. *Nat. Genet.* **37,** 1309–1311 (2005).
7 Karim, M. A., Parsian, A J., Cleves, M. A., Bracey, J., Elsayed, M. S., Elsobky, E. *et al.* A novel mutation in BAP/SIL1 gene causes Marinesco-Sjögren syndrome in an extended pedigree. *Clin. Genet.* **70,** 420–423 (2006).
8 Eriguchi, M., Mizuta, H., Kurohara, K., Fujitake, J. & Kuroda, Y. Identification of a new homozygous frameshift insertion mutation in the SIL1gene in 3 Japanese patients with Marinesco-Sjögren syndrome. *J. Neurol. Sci.* **270,** 197–200 (2008).
9 Anttonen, A. K., Siintola, E., Tranebjaerg, L., Iwata, N. K., Bijlsma, E. K., Meguro, H. *et al.* Novel SIL1 mutations and exclusion offunctional candidate genes in Marinesco-Sjögren syndrome. *Eur. J. Hum. Genet.* **16,** 961–969 (2008).
10 Annesi, G., Aguglia, U., Tarantino, P., Annesi, F., De Marco, E. V., Civitelli, D. *et al.* SIL1 and SARA2 mutations in Marinesco-Sjögren and chylomicron retention diseases. *Clin. Genet.* **71,** 288–289 (2007).
11 Zhao, L., Longo-Guess, C., Harris, B. S., Lee, J. W. & Ackerman, S.L. Protein accumulation and neurodegeneration in the woozy mutant mouse is caused by disruption of SIL1, a cochaperone of BiP. *Nat Genet.* **37,** 974–979 (2005).
12 Weitzmann, A., Volkmer, J. & Zimmermann, J. The nucleotide exchange factor activity of Grp170 may explain the non-lethal phenotype of loss of Sil1 function in man and mouse. *FEBS Lett.* **580,** 5237–5240 (2006).
13 Weitzmann, A., Baldes, C., Dudek, J. & Zimmermann, R. The heat shock protein 70 molecular chaperone network in the pancreatic endoplasmic reticulum. *FEBS J.* **274,** 5175–5187 (2007).

Human Reproduction, Vol.25, No.4 pp. 1076–1080, 2010
Advanced Access publication on February 6, 2010 doi:10.1093/humrep/deq006

human reproduction CASE REPORT *Reproductive genetics*

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

Kiyonori Miura[1,*], Shoko Miura[1], Koh-ichiro Yoshiura[2], Stephanie Seminara[3], Daisuke Hamaguchi[1], Norio Niikawa[4], and Hideaki Masuzaki[1]

[1]Department of Obstetrics and Gynecology, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki, Japan [2]Department of Human Genetics, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan [3]Reproductive Endocrine Unit, Massachusetts General Hospital, Boston, MA 02114, USA [4]Research Institute of Personalized Health Sciences, Health Sciences University of Hokkaido, Hokkaido, Japan

[*]Correspondence address. Tel: +81-95-819-7363; Fax: +81-95-819-7365; E-mail: kiyonori@nagasaki-u.ac.jp

Submitted on August 24, 2009; resubmitted on December 17, 2009; accepted on January 4 2010

ABSTRACT: Fibroblast growth factor receptor 1 (*FGFR1*) is one of the causative genes for Kallmann syndrome (KS), which is characterized by isolated hypogonadotropic hypogonadism with anosmia/hyposmia. The third immunoglobulin-like domain (D3) of *FGFR1* has the isoforms *FGFR1-IIIb* and *FGFR1-IIIc*, which are generated by alternative splicing of exons 8A and 8B, respectively. To date, the only mutations to have been identified in D3 of *FGFR1* are in exon 8B. We performed mutation analysis of *FGFR1* in a 23-year-old female patient with KS and found a missense mutation (c.1072C>T) in exon 8A of *FGFR1*. The c.1072C>T mutation was not detected in her family members or in 220 normal Japanese and 100 Caucasian female controls. No mutation in other KS genes, KS 1, prokineticin-2, prokineticin receptor-2 and *FGF-8* was detected in the affected patient or in her family members. Therefore, this is the first case of KS carrying a *de novo* missense mutation in *FGFR1* exon 8A, suggesting that isoform *FGFR1-IIIb*, as well as isoform *FGFR1-IIIc*, plays a crucial role in the pathogenesis of KS.

Key words: Kallmann syndrome / *FGFR1b mutation* / fibroblast growth factor receptor 1 isoform expression

Introduction

Kallmann syndrome (KS), which is characterized by isolated hypogonadotropic hypogonadism (IHH) and anosmia/hyposmia, is a clinically and genetically heterogeneous disorder. To date, five causative genes for KS have been reported: KS 1 (*KAL1*, GenBank accession M97 252), prokineticin-2 (*PROK2*, GenBank accession NM 021935), prokineticin receptor-2 (*PROKR2*, GenBank accession NM 144773), fibroblast growth factor-8 (*FGF-8*, GenBank accession NM 033163) and fibroblast growth factor receptor 1 (*FGFR1*, GenBank accession NM 023110.2).

Although sporadic cases of KS are more frequent, families with KS have been reported with X-linked recessive or autosomal dominant or recessive modes of inheritance. Mutations in *KAL1* have been found in familial cases with X-linked recessive inheritance (Franco et *al.*, 1991; Legouis et *al.*, 1991). Mutations in *PROK2* were detected in the heterozygous state, whereas *PROKR2* mutations were found in the heterozygous, homozygous or compound heterozygous state (Dodé et *al.*, 2006). *PROKR2/PROK2* mutations with true pathogenic potential were found only in the homozygous state (Abreu et *al.*, 2008), and any dominant-negative effect of *PROKR2* mutations was ruled out (Monnier et *al.*, 2009). Mutations in *FGFR1* or *FGF8* underlie an autosomal dominant form with incomplete penetrance. Therefore, KS families harbouring heterozygous *FGFR1* or *FGF8* mutations display variable olfactory phenotypes (Dodé et *al.*, 2003; Falardeau et *al.*, 2008), and a few cases with heterozygous *FGFR1* mutations show a normosmic IHH (Pitteloud et *al.*, 2006a). The *FGFR1* gene, which is located on chromosome 8p12, comprises 18 exons (Ruta et *al.*, 1989), and various mutations, including missense and protein truncation mutations, have been reported (Trarbach et *al.*, 2007). The third immunoglobulin-like domain (D3) of *FGFR1* has the isoforms *FGFR1-IIIb* and *FGFR1-IIIc*, which are generated by alternative splicing

of exons 8A and 8B, respectively (Johnson *et al.*, 1991). To date, mutations in D3 of *FGFR1* have only been identified in exon 8B, which encodes immunoglobulin domain IIIc, suggesting that isoform FGFR1-IIIc plays a crucial role in the pathogenesis of KS (Pitteloud *et al.*, 2006b; Trarbach *et al.*, 2006; Dodé *et al.*, 2007).

Here, we report for the first time a KS case carrying a *de novo* missense mutation in the alternatively spliced exon 8A of *FGFR1-IIIb*.

Materials and Methods

Patient and family

Patient (Subject II-2) was a 23-year-old Japanese woman. When she was 18 years old, she was treated at Nagasaki University Hospital because of primary amenorrhea with anosmia. Her height was 159.2 cm and her weight was 72.0 kg. Her serum levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH) and estradiol (E2) were less than 0.5 and 1.5m IU/ml and 10 pg/ml, respectively. Her LH frequent sampling study (sampling performed every 15 min) showed a low-amplitude pattern of LH pulsation (Fig. 1). Her brain magnetic resonance imaging (MRI) examination was negative for tumors and showed no anatomical abnormalities of the hypothalamic–pituitary region and olfactory bulbs. A scratch-and-sniff test (UPSIT, Sensonics, Haddon Hts, NJ, USA) (Doty *et al.*, 1985), which determines ability to smell, indicated anosmia. She was diagnosed as having KS and received hormone replacement therapy for 5 years. Her mother (Subject I-1) was normosmic and had normal puberty and regular menstrual cycles. Her father (Subject I-2), elder brother (Subject II-1) and younger brother (Subject II-3) were also normosmic and had normal puberty (Fig. 2).

Molecular analysis

DNA extraction

Whole blood samples were obtained from the KS patient and from her mother, father, elder and younger brothers. All samples were collected after obtaining written informed consent and the study protocol was approved by the Institutional Review Board of Nagasaki University. Genomic DNA from lymphocytes was extracted using a QIAamp DNA blood mini kit (Qiagen, Düsseldorf, Germany), according to the manufacturer's instructions.

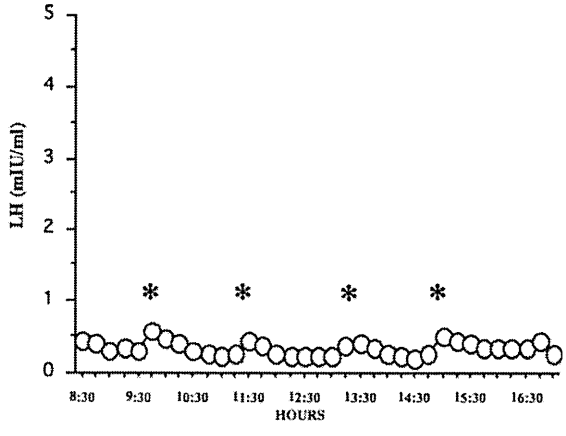

Figure 1 LH pulsation pattern in a case of KS, assayed using an LH frequent sampling study. LH frequent sampling was performed every 15 min. *Low-amplitude pattern of LH pulse.

Sequence analysis

FGFR1 consists of 18 coding exons. Intragenic mutations were investigated by PCR amplification and sequence analysis using 14 pairs of primers, as previously described (Dodé *et al.*, 2003; Sato *et al.*, 2004). Genomic DNA was PCR amplified using conditions of 95°C for 12 min followed by 95°C for 30 s, 59°C for 30 s and 72°C for 60 s for 35 cycles and a final cycle of 72°C for 10 min. PCR products were analyzed by agarose gel electrophoresis, purified with ExoSAP-IT and subjected to sequencing reactions. Sequencing reactions were performed using the BigDye terminator v.3.1 kit and analyzed with an ABI PRISM 3100 Genetic Analyzer™ (Applied Biosystems). The KS patient carrying a mutation in *FGFR1* and her family members were also screened for mutations in the other genes known to be involved in KS [*KAL1*, *PROK2*, *PROKR2* and *FGF8*]. Whether the mutation leads to a change in the protein structure and function was predicted bioinformatically using the ExPASy proteomics server (http://au.expasy.org/) and PolyPhen (http://genetics.bwh.harvard.edu/pph/).

Confirmation of the alternatively spliced exon

Isolation of a full-length murine *Fgfr1-IIIb* showed that Fgfr1-IIIb was a transmembrane receptor (Beer *et al.*, 2000). Although the mRNA encoding exon IIIb has been found in human (Johnson *et al.*, 1991), the presence of sequences encoding the intracellular domain has not yet been demonstrated. Therefore, to determine the splice site of exon 8A and to detect *FGFR1-IIIb* mRNA encoding the intracellular domain, we performed RT–PCR using specific primers to amplify the splice isoform containing exon 8A. Kal23 is designed to span exons 7 and 8A for specific annealing to the *FGFR1-IIIb* isoform, which is spliced from exon 7 to exon 8A (Fig. 3A). Kal5 is designed within exon 8B for specific annealing to the *FGFR1-IIIc* isoform, which is spliced from exon 7 to exon 8B. Kal2 and Kal6 are designed within exons 7 and 9, respectively, for annealing to the D3 isoforms of *FGFR1*. Primer sequences were as follows: kal2: 5′-GACAGAAGGTCGGTTATGTC-3′, Kal23: 5′-CAGATCTTGAAGC ATTCGGG-3′, Kal5: 5′-GGTGGTATTAACTCCAGCAG-3′ and Kal6: 5′-GTACAGGGGCGAGGTCATCA-3′. The BD multiple tissue complementary DNA (cDNA, MTC) panels Human I and Human II (BD Biosciences Clontech, Mountain View, CA, USA) were used to detect the expression of each isoform of *FGFR1*. PCR amplification was performed on cDNAs as follows: 94°C, 30 s; 62°C, 30 s; 72°C, 1 min; 40 cycles. PCR products were analyzed by agarose gel electrophoresis and sequenced using then ABI PRISM 3100 Genetic Analyzer™.

Results

Sequence analysis of the entire coding region of *FGFR1*, including exon–intron boundary regions, showed that the KS patient had a mutation (c.1072C>T) in exon 8A of *FGFR1-IIIb*, while the other family members did not (Fig. 2). However, the full-length *FGFR1* mRNA that includes exon 8A is not deposited in the full-length cDNA database (GenBank accession no. NM 023110.2). RT–PCR analysis indicated that most transcripts containing exon 8A were spliced to exon 8B in all adult tissues except bone marrow (data not shown). We wished to demonstrate the existence of an alternative transcript, exon 8A which was spliced to exon 9 encoding the transmembrane helix; therefore, RT–PCR products from human fetal brain were cloned and sequenced. In 1 of 27 clones exon 8A was spliced to exon 9 (designated here '*FGFR1-IIIb*', GenBank accession FJ809917, see Fig. 3B), while in the other clones exon 8A was spliced to exon 8B (designated here

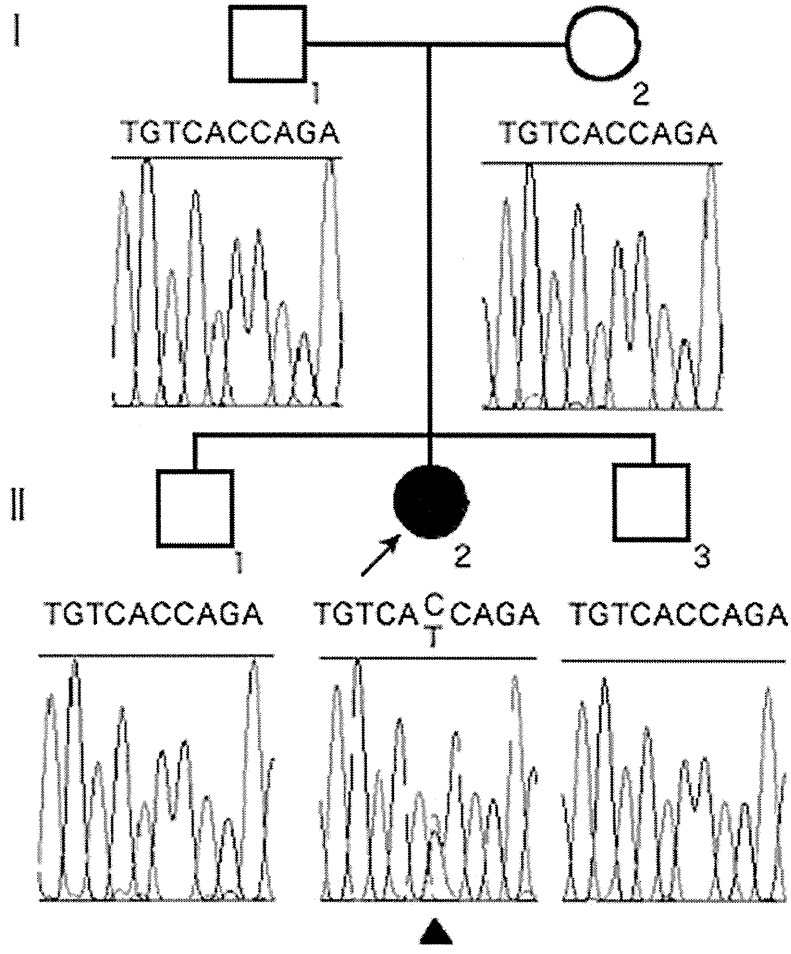

Figure 2 Pedigree of patient's family and the results of fibroblast growth factor receptor 1 (*FGFR1*) sequence analysis. II-2 is a 23-year-old woman with KS. The patient had a mutation in *FGFR1* (C>T) but the other family members did not. The mutation was *de novo* because parentage was assured. Arrowhead under the electropherogram indicates the mutation site.

'*FGFR1-secr*', GenBank accession FJ809916, see Fig. 3B). The exact acceptor and donor sites of exon 8A in '*FGFR1-IIIb*' mRNA, which produces a membrane-bound FGFR1-containing D3, were determined by sequence analysis of splice isoforms, '*FGFR1-IIIb*' and '*FGFR1-secr*', (Fig. 3). As most full-length *FGFR1* cDNAs in the database were transcripts containing exon 7–exon 8B–exon 9 (designated here '*FGFR1-IIIc*', GenBank accession NM 023110.2, see Fig. 2B) without exon 8A, '*FGFR1-IIIc*' is likely to be the most abundantly expressed human isoform. The EST, CA488712.1, was the only isoform in the EST database corresponding to '*FGFR1-IIIb*'. Although both '*FGFR1-IIIb*' and '*FGFR1-IIIc*' encode membrane-bound *FGFR1*, '*FGFR1-secr*' encodes a secreted form of *FGFR1* because of a sequence frameshift and a termination codon in exon 9.

The exons 8A and 8B of the human FGFR1 isoforms shared the amino acid sequence at 354–357; WLTV. However, exon 8A ends with six extra amino acids at 358–363, TRPVAK, whereas exon 8B ends with only two, LE. These sequences are identical in the mouse Fgfr1 isoforms (Beer *et al.*, 2000). The mutation in exon 8A of *FGFR1-IIIb* (GenBank accession no. FJ809917 bankit1193625) is c.1072C>T at the cDNA level and p.T358I at the amino acid level. Bioinformatic analysis shows the mutated amino acid residue to be conserved between human and mouse and to be located in D3 of *FGFR1-IIIb*, which is a critical region for FGF ligand binding. However, the mutation was not predicted to produce a change in the human protein structure. The c.1072C>T mutation was not detected in 220 normal Japanese women or in 100 normal Caucasian women. The patient had no mutation in any of the other four KS genes.

Discussion

Mouse *Fgfr1-IIIb* has a low level of expression in a wide variety of adult tissues, but a high level of expression in skin and brain, indicating the existence of specific splicing factors in skin and brain that recognize the relatively weak *Fgfr1-IIIb* splice site (Beer *et al.*, 2000). Consistent with the expression pattern of mouse *Fgfr1-IIIb*, we could isolate human *FGFR1-IIIb* from a fetal brain cDNA library but not from adult

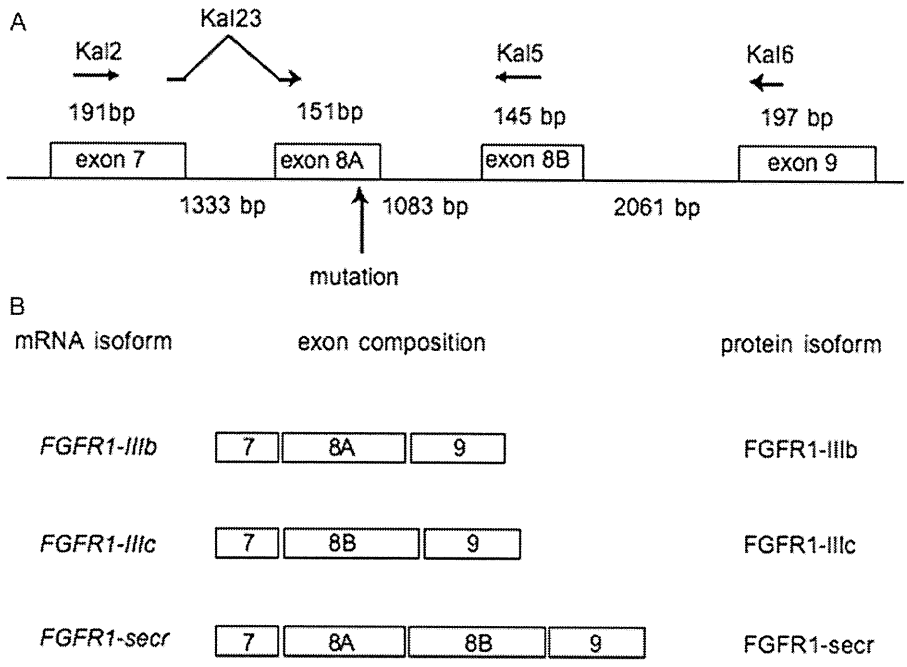

Figure 3 Genomic organization of *FGFR1* around exon 8A. (A) The numbers with bp indicate exon length (over the line) and intron length (under the line). The mutation is located near the end of exon 8A. Horizontal arrows indicate the locations of primers used to perform RT–PCR amplification of the isoform containing exons 7 and 8A. Primer Kal23 is designed to span exons 7 and 8A for specific annealing to the *FGFR1-IIIb* isoform, which is spliced from exon 7 to exon 8A (Fig. 3A). Kal5 is designed within exon 8B for specific annealing to the *FGFR1-IIIc* isoform, which is spliced from exon 7 to exon 8B. Kal2 and Kal6 are designed within exons 7 and 9, respectively, for annealing to the D3 isoforms of *FGFR1*. Vertical arrow indicates mutation site. (B) Composition of mRNA isoforms and of putative protein structures. FGFR1-IIIb: membrane-bound form of FGFR1 with immunoglobulin-like domain IIIb encoded by exon 8A, FGFR1-IIIc: membrane-bound form of FGFR1 with immunoglobulin-like domain IIIc encoded by exon 8B, FGFR1-secr: a secreted form of FGFR1.

tissues. Most full-length *FGFR1* cDNAs in the database represent *FGFR1-IIIc*. *FGFR1-IIIc* is expressed at high levels, but *FGFR1-IIIb* is expressed at very low levels (Johnson *et al.*, 1991). We can amplify only a tiny amount of *FGFR1-IIIb* that has exon 8A spliced to exon 9 by RT–PCR using the primers Kal23 and Kal6. Most of the sequenced RT–PCR products corresponded to *FGFR1-secr*, suggesting that the expression level of the three isoforms is '*FGFR1-IIIc*' ≫ '*FGFR1-IIIsecr*' ≫ '*FGFR1-IIIb*' (Fig. 3B).

Several studies suggested that mutations in exon 8B of isoform *FGFR1-IIIc* are implicated in the pathogenesis of KS (Pitteloud *et al.*, 2006b; Trarbach *et al.*, 2006; Dodé *et al.*, 2007). Mice homozygous for alleles with a stop codon in exon IIIc displayed phenotypes resembling those of embryos homozygous for null alleles, while mice carrying an in-frame stop codon in exon IIIb were viable and fertile (Partanen *et al.*, 1998). Therefore, *Fgfr1-IIIc* is the dominant isoform that carries out the majority of the biological functions of the *Fgfr1* gene, whereas *Fgfr1-IIIb* plays a minor and to some extent redundant role (Partanen *et al.*, 1998). A receptor-binding analysis revealed no difference in the binding specificity between the endogenous *Fgfr1-IIIb* and an artificially created *Fgfr1-IIIb*, which had two different amino acids in the 3′-end of the unique IIIb exon (Beer *et al.*, 2000), suggesting that the carboxyl terminus of D3 may not overtly influence binding specificity. However, being expressed at low levels does not imply that '*FGFR1-IIIb*' has an unimportant role. The mutation we found, p.T358I, was located in exon 8A of *FGFR1-IIIb*. Therefore, p.T358I may affect ligand binding and cause the KS phenotype, although how this mutation affects the loss of function of *FGFR1-IIIb* is unknown. The spatio-temporal expression of any gene involved in development is key; therefore, the expression and functional involvement of *FGFR1-IIIb* may be important in the early embryonic brain, in particular during GnRH neuronal development. KS missense mutations in *FGFR1* are distributed in the first, second and third immunoglobulin-like domains (D1–D3), in the tyrosine kinase domain and also in the intracellular domain (Dodé *et al.*, 2003; Sato *et al.*, 2004; Albuisson *et al.*, 2005; Pitteloud *et al.*, 2006a; Trarbach *et al.*, 2006; Dodé *et al.*, 2007); therefore, the membrane-bound form of *FGFR1* is probably important for the KS phenotype. The membrane-bound form of *FGFR1-IIIb* could, therefore, be the critical isoform for the KS phenotype.

We present here, for the first time, a case of KS carrying a missense mutation in exon 8A of *FGFR1*, suggesting that the minor isoform '*FGFR1-IIIb*' as well as the major isoform '*FGFR1-IIIc*' has a crucial role in the pathogenesis of KS. Therefore, immunoglobulin-like domain IIIb may have an essential role in GnRH neuronal migration, which is initiated from the nasal placode and runs towards the forebrain following the olfactory sensory neuron axonal connection with the developing olfactory bulb. Further experiments are needed to show that the mutation in

exon 8A causes KS, such as expression of the mutated isoform in transfected cells to analyze receptor stability and signaling efficiency. Although *FGFR1* containing immunoglobulin-like domain IIIb has not been analyzed intensively, our mutation report should encourage researchers to analyze immunoglobulin-like domain IIIb function and the spatio-temporal expression of exon 8A in fetal brain development.

Acknowledgements

We thank Ms Yasuko Noguchi and Miho Ooga for their technical assistance and we thank Drs Nelly Pitteloud and William Crowley for their valuable contribution.

Funding

K.M. was supported, in part, by Seeds (No.15-B09) from the Japan Science and Technology Agency (JST), by grants from the Naito Foundation, by Grant-in-Aid for Young Scientists (B) (no. 21791567) from the Ministry of Education, Sports, Culture, Science and Technology of Japan, by a Grant for Child Health and Development (20C-1) from the Ministry of Health, Labor and Welfare, and by Grant-in-Aid for Scientific Research from Nagasaki University, Japan.

References

Abreu AP, Trarbach EB, de Castro M, Frade Costa EM, Versiani B, Matias Baptista MT, Garmes HM, Mendonca BB, Latronico AC. Loss-of-function mutations in the genes encoding prokineticin-2 or prokineticin receptor-2 cause autosomal recessive Kallmann syndrome. *J Clin Endocrinol Metab* 2008;**93**:4113–4118.

Albuisson J, Pêcheux C, Carel JC, Lacombe D, Leheup B, Lapuzina P, Bouchard P, Legius E, Matthijs G, Wasniewska M *et al.* Kallmann syndrome: 14 novel mutations in KAL1 and FGFR1 (KAL2). *Hum Mutat* 2005;**25**:98–99.

Beer HD, Vindevoghel L, Gait MJ, Revest JM, Duan DR, Mason I, Dickson C, Werner S. Fibroblast growth factor (FGF) receptor 1-IIIb is a naturally occurring functional receptor for FGFs that is preferentially expressed in the skin and the brain. *J Biol Chem* 2000; **275**:16091–16097.

Dodé C, Levilliers J, Dupont JM, De Paepe A, Le Dû N, Soussi-Yanicostas N, Coimbra RS, Delmaghani S, Compain-Nouaille S, Baverel F *et al.* Loss-of-function mutation in *FGFR1* cause autosomal dominant Kallmann syndrome. *Nature Genet* 2003;**33**:463–465.

Dodé C, Teixeira L, Levilliers J, Fouveaut C, Bouchard P, Kottler ML, Lespinasse J, Lienhardt-Roussie A, Mathieu M, Moerman A *et al.* Kallmann syndrome: mutations in the genes encoding prokineticin-2 prokineticin receptor-2. *PLoS Genet* 2006;**2**:e175.

Dodé C, Fouveaut C, Mortier G, Janssens S, Bertherat J, Mahoudeau J, Kottler ML, Chabrolle C, Gancel A, François I *et al.* Novel FGFR1 sequence variants in Kallmann syndrome, and genetic evidence that the FGFR1c isoform is required in olfactory bulb and palate morphogenesis. *Hum Mutat* 2007;**28**:97–98.

Doty RL, Applebaum S, Zusho H, Settle RG. Sex differences in odor identification ability: a cross-cultural analysis. *Neuropsychologia* 1985; **23**:667–672.

Falardeau J, Chung WC, Beenken A, Raivio T, Plummer L, Sidis Y, Jacobson-Dickman EE, Eliseenkova AV, Ma J, Dwyer A *et al.* Decreased FGF8 signaling causes deficiency of gonadotropin-releasing hormone in humans and mice. *J Clin Invest* 2008;**118**:2822–2831.

Franco B, Guioli S, Pragliola A, Incerti B, Bardoni B, Tonlorenzi R, Carrozzo R, Maestrini E, Pieretti M, Taillon-Miller P *et al.* A gene deleted in Kallmann's syndrome shares homology with neural cell adhesion and axonal path-finding molecules. *Nature* 1991;**353**:529–536.

Johnson DE, Lu J, Chen H, Werner A, Williams LT. The human fibroblast growth factor receptor genes: a common structure arrangement underlies the mechanisms for generating receptor forms that differ in their third immunoglobulin domain. *Mol Cell Biol* 1991;**11**:4627–4634.

Legouis R, Hardelin JP, Levilliers J, Claverie JM, Compain S, Wunderle V, Millasseau P, Le Paslier D, Cohen D, Caterina D *et al.* The candidate gene for the X-linked Kallmann syndrome encodes a protein related to adhesion molecules. *Cell* 1991;**67**:423–435.

Monnier C, Dodé C, Fabre L, Teixeira L, Labesse G, Pin JP, Hardelin JP, Rondard P. PROKR2 missense mutations associated with Kallmann syndrome impair receptor signalling activity. *Hum Mol Genet* 2009; **18**:75–81.

Partanen J, Schwartz L, Rossant J. Opposite phenotypes of hypomorphic and Y766 phosphorylation site mutations reveal a function for Fgfr1 in anteroposterior patterning of mouse embryos. *Genes Dev* 1998; **12**:2332–2344.

Pitteloud N, Acierno JS Jr, Meysing A, Eliseenkova AV, Ma J, Ibrahimi OA, Metzger DL, Hayes FJ, Dwyer AA, Hughes VA *et al.* Mutations in fibroblast growth factor receptor 1 cause both Kallmann syndrome and normosmic idiopathic hypogonadotropic hypogonadism. *Proc Natl Acad Sci USA* 2006a;**103**:6281–6286.

Pitteloud N, Meysing A, Quinton R, Acierno JS Jr, Dwyer AA, Plummer L, Fliers E, Boepple P, Hayes F, Seminara S *et al.* Mutations in fibroblast growth factor receptor 1 cause Kallmann syndrome with a wide spectrum of reproductive phenotypes. *Mol Cell Endocrinol* 2006b; **254–255**:60–69.

Ruta M, Burgess W, Givol D, Epstein J, Neiger N, Kaplow J, Crumley G, Dionne C, Jaye M, Schlessinger J. Receptor for acidic fibroblast growth factor is related to the tyrosine kinase encoded by the fms-like gene (FLG). *Proc Natl Acad Sci USA* 1989;**86**:8722–8726.

Sato N, Katsumata N, Kagami M, Hasegawa T, Hori N, Kawakita S, Minowada S, Shimotsuka A, Shishiba Y, Yokozawa M *et al.* Clinical assessment and mutation analysis of Kallmann syndrome 1 (KAL1) and fibroblast growth factor receptor 1 (FGFR1, or KAL2) in five families and 18 sporadic patients. *J Clin Endorinol Matab* 2004; **89**:1079–1088.

Trarbach EB, Costa EM, Versiani B, de Castro M, Baptista MT, Garmes HM, de Mendonca BB, Latronico AC. Novel fibroblast growth factor receptor 1 mutations in patients with congenital hypogonadotropic hypogonadism with and without anosmia. *J Clin Endocrinol Metab* 2006;**91**:4006–4012.

Trarbach EB, Silveira LG, Latronico AC. Genetic insights into human isolated gonadotropin deficiency. *Pituitary* 2007;**10**:381–391.