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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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, Senderek *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- μ l reaction mixture containing 30 ng genomic DNA, 0.5 μ M each of forward and reverse primers, 200 μ M each of dNTP in 1 $\times$ 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- μ l 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 μ g 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- μ m 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- μ l reaction mixture containing 10 ng genomic DNA, 0.5 μ M each of forward and reverse primers, 200 μ M each of dNTP, 1 $\times$ ExTaq buffer, 0.2 μ M 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'-AGCGATCAGTAAGGGTATT-3' for SILint5-delF and 5'-CAGTGTCTGGAAGCACAAAGC-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 *SIL1* 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 (<i>SIL1</i> exon 2)	
Forward primer	5'-CTCTTGTGGATGGCTGGAC-3'
Reverse primer	5'-TGTGATTCCCATGTCGTCAC-3'
Target sequence (<i>SIL1</i> exon 6)	
Forward primer	5'-GGCAGATGTCTCCAACCAAT-3'
Reverse primer	5'-CTTGTGATCAGCCGTACCA-3'
Target sequence (<i>SIL1</i> exon 10)	
Forward primer	5'-AGAGCTAGCCAGGTGTGAGC-3'
Reverse primer	5'-AGGAGGTGTACCTGGCGATA-3'
Reference sequence (<i>NSD1</i>)	
Forward primer	5'-ATGCTTTTTAGCCCAATG-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.

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