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G. 知的財産権の出願・登録状況（予定を含む）

1. 特許取得

該当なし。

2. 実用新案登録

該当なし。

3. その他

該当なし。

平成２３年度厚生科学研究費補助金（障害者対策総合研究事業）
分担研究報告書

超細密染色体分析から捉え直すヒト発達障害研究

分担研究課題：年齢依存性てんかん性脳症の遺伝型と臨床型の関連性の解明

分担研究者　　加藤光広　　山形大学医学部小児科学講座

研究要旨：
シナプス小胞の膜結合に関与する *STXBP1* は大田原症候群の原因遺伝子である。大田原症候群 14 例，ウエスト症候群 20 例、その他の乳児期発症てんかん性脳症 10 例に対して *STXBP1* の変異解析を行い，病的変異を示した 7 例（大田原症候群５例、ウエスト症候群１例、分類不能の乳児期発症てんかん性脳症１例）の臨床型を解析した。全例経過中にスパズム発作をきたしたが、初期症状はさまざまであった。3 例は ACTH で、1 例は PB 大量療法で発作が消失したが、全例重度の発達遅滞を呈した。*STXBP1* 変異は大田原症候群の主要な原因であり、一部は大田原症候群を介さないウエスト症候群の原因となる。

Ａ．研究目的

年齢依存性てんかん性脳症は，てんかんに加え認知機能や運動障害などの発達障害を併発する疾患であり，発達期に応じた特徴的な発作型や脳波所見によって分類される複数のてんかん症候群で構成されている。新生児期に発症する大田原症候群と乳児期に発症するウエスト症候群は，年齢依存性てんかん性脳症の代表的疾患である。私たちは 2008 年に *STXBP1* が大田原症候群の原因遺伝子であることを明らかにしたが，その後の欧州からの報告では，*STXBP1* 変異は非特異的な乳幼児期発症のてんかん性脳症やウエスト症候群で同定され，大田原症候群では同定されないなど，*STXBP1* 変異の臨床像がまだ十分明らかになっていない。

本研究では，STXBP1 遺伝子変異による臨床型を明らかにする事を目的とした。

Ｂ．研究方法

保護者の承諾を得て，脳形成異常や代謝異常，周産期障害による明らかな原因を除外された年齢依存性てんかん性脳症の患者 44 例（大田原症候群 14 例，ウエスト症候群 20 例，その他の乳児期発症てんかん性脳症 10 例）および両親の血液から DNA を抽出し，さらに臨床情報（発作型・脳波・頭部 MRI・使用薬剤と効果・併発症など）を収集した。

STXBP1 遺伝子の全コード領域を high resolution melting (HRM)法を用いて変異スクリーニングを行い，陽性例に対して直接塩基配列の解析を行った。なお，男性では *ARX* 遺伝子を，女性では *CDKL5* 遺伝子の変異スクリーニングを行い，それぞれの変異例は除外した。

Ｃ．研究結果

大田原症候群５例、ウエスト症候群１例、分類不能の乳児期発症てんかん性脳症１例の計７例において *STXBP1* 変異を同定した。内訳はナンセンス変異３例，スプライシング異常２例，ミスセンス変異２例で，両親の検体が得られていない１例を除き全例新生変異であった。

７例の年齢は 2-13 か月（平均 6.4 か月）で、女児５例、男児２例であった。発症は生後１日から５か月（平均 1.6 か月）で、全例経過中にスパズム発作を呈したが、初期からスパズム発作をきたしたのは１例のみで、初発発作としては嘔吐や息止め、眼球上転など一定しなかった。大田原症候群の２例を除き５例がヒプスアリスミアを伴うウエスト症候群に変容し、平均 3.2 か月で移行していた。4 例(ACTH 3 例，高用量フェノバルビタール 1 例)は治療に反応し発作が一時的に消失したが，3 例は反応せず１日に数回発作がみられた。全例重度の発達遅滞を呈し、１例は体幹筋緊張の亢進が著明で後弓反張を呈した。乳児早期（1〜4 か月)に撮影された６例の MRI は正常であったが，9 か月に撮影された１例では脳萎縮と硬膜下血腫を認めた。

Ｄ．考察

今回始めてサプレッション・バーストを示さな

い２症例において *STXBP1* 変異を認め、他の報告同様 *STXBP1* が大田原症候群以外の表現型をきたすことが確認された。２例は発症が２か月、３か月と他に比べて遅いが、ヒプスアリスミアを伴うスパズム発作を呈し、ACTH が発作消失に有効であった。大田原症候群における変異頻度は 36%と既報告通り高率だが、ウエスト症候群では前回スクリーニングした 54 例と合わせると 1.4%であり、大田原症候群に比較してまれであった。遺伝型による表現型の差はなく、ハプロ不全を示唆する。

ACTH 以外に１例でフェノバルビタールの大量療法が有効性を示した。大田原症候群におけるフェノバルビタール大量療法の有効性は経験的に知られていたが、今後基礎原因との関連性を明らかにする必要がある。

Ｅ．結論

STXBP1 変異は大田原症候群の主要な原因であり、一部は大田原症候群を介さないウエスト症候群の原因となる。

Ｆ．健康危険情報

特になし。

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Ｈ．知的財産権の出願・登録状況

なし

平成２３年度厚生科学研究費補助金（障害者対策総合研究事業）
分担研究報告書

超細密染色体分析から捉え直すヒト発達障害研究

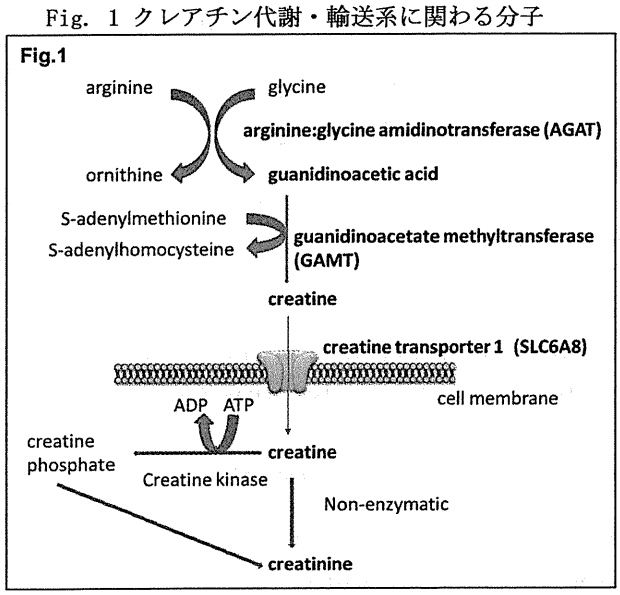
分担研究課題：本邦におけるクレアチン欠損症のスクリーニング法確立に向けて
分担研究者　　小坂仁　　神奈川県立こども医療センター神経内科
研究協力者　　和田敬仁　　神奈川県立こども医療センター神経内科

研究要旨：クレアチン欠損症は L-arginine: glycine amidinotransferase 欠損症, guanidinoacetate methyltransferase 欠損症, creatine transporter 欠損症よりなる。欧米では精神遅滞の 2%前後を占めるとも言われるが、本邦での報告例はなく、その頻度も不明である。クレアチン欠損症の診断法としては、高速クロマトグラフィーと質量分析計を用いた測定法が用いられ、一般的な普及となっていない。我々は、HPLC を用いた簡便なスクリーニング法を開発し、この方法を用い当院の発達遅滞患者にスクリーニングを行い、本邦初症例となる２家系を見出した。

A．研究目的

クレアチン(CR)は、生体内において ATP の速やかな産生に重要な役割を持つことが、近年明らかになりつつある。特に脳において CR は、血液脳関門を通過しないために、独自の合成、輸送経路を持つことが知られる。これらの異常により、脳性クレアチン欠損症；Cerebral creatine deficiency syndromes, CCDS を呈する。現在までに(L-arginine: glycine amidinotransferase (AGAT)欠損症, guanidinoacetate methyltransferase (GAMT) 欠損症, creatine transporter (SLC6A8) が知られる(Fig. 1)。

Fig. 1 クレアチン代謝・輸送系に関わる分子



これら 3 疾患の共通の症状は精神遅滞、自閉症、てんかんであり、欧米では精神遅滞の 2%前後を占めるといわれるが、本邦では報告例がなく、その頻度は不明である。

この疾患のスクリーニング方法としては、高速クロマトグラフィーと質量分析を組み合わせた方法が診断の主流であるが、使用できる施設が限られており、昨年 HPLC を用いた簡便なスクリーニング方法を開発し報告した。今年度は、この方法を用いて、本邦でのスクリーニングの初年度の結果を報告する。

B．研究方法

対象は、当院通院中の原因不明の精神運動発達遅滞児(1-15 才)であり、原則としてアミノ酸代謝異常、染色体検査、甲状腺機能、乳酸・ピルビン酸異常を除外された症例である。凍結尿 500 µl を等量のアセトニトリル添加後、遠心分離し、25 µl を用い、CR：クレアチン、 GAA：グアニジノ酢酸、GN：クレアチニンを測定し、年齢平均の 2SD の高値あるいは低値をもって一次スクリーニング陽性とした。(参照、Table 1; AGAT 欠損症; GAA/CN↓CR/CN↓、GAMT 欠損症;GAA/CN↑ CR/CN↓、SLC6A8 欠損症；GAA/CN 正常、CR/CN↑)。このスクリーニングにより、異常が疑われた患者については、同意を所得後、患者血液から DNA を採取し、それぞれ原因遺伝子である *AGAT, GAMT, SLC6A8* 遺伝子を PCR ダイレクトシークエンスにより塩基配列を決定した。

Table 1. Urine Creatine and Guanidinoacetic acid Levels in Patients with Defects of Creatine Synthesis and Transport

Disease	GA/CN	CR/CN
AGAT deficiency	Low	Low
GAMT deficiency	High	Normal
CR transporter deficiency	Normal	High (4017.5±286.4)

( ); values from one patient with CR transporter deficiency (μmol/mmol); HPLC method; 4017.5±286.4; mean ±SD, n=2)
Normal values with this study from 15 samples are 798.6±574.8(CR/CN(μmol/mmol)(HPLC method; mean ±SD)

Abbrebiations;arginine: glycine amidinotransferase, AGAT; guanidinoacetate methyltransferase, GAMT; creatine, CR; Guanidinoacetic acid, GA; Creatinine, CN;

Table 1. 尿代謝物によるクレアチン代謝異常スクリーニング

C．研究結果

①当院の精神遅滞症例98名のスクリーニングを行いAGAT；1名，GAMT；2名，SLC6A8；5名の対象者が、スクリーニング陽性とされた。

②これらの8名で、塩基配列を決定したところ、2名でSLC6A8異常（1例は全欠失）を確認した。いずれの患者も磁気共鳴分光法で大脳におけるCRピークの消失が消失していた。

D．考察

本邦初症例となるSLC6A8異常症2例を見いだした。いままで日本でCCDSの報告がないのは、この疾患の存在自体が知られていないことや、測定が難しい事などに起因する可能性がある。

E．結論

本邦でもCCDS欠損、特にSLC6A8欠損症例が存在することがわかった、女児においては大量のクレアチンが臨床症状を改善することが知られており、治療可能な発達遅滞として今後本邦でのスクリーニング体制を確立する必要がある。

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H. 知的所有権の取得状況；なし

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Short Report

Paternal mosaicism of an *STXBP1* mutation in OS

Saitsu H, Hoshino H, Kato M, Nishiyama K, Okada I, Yoneda Y, Tsurusaki Y, Doi H, Miyake N, Kubota M, Hayasaka K, Matsumoto N. Paternal mosaicism of an *STXBP1* mutation in OS. Clin Genet 2011: 80: 484–488. © John Wiley & Sons A/S, 2010

Ohtahara syndrome (OS) is one of the most severe and earliest forms of epilepsy. We have recently identified that the *de novo* mutations of *STXBP1* are important causes for OS. Here we report a paternal somatic mosaicism of an *STXBP1* mutation. The affected daughter had onset of spasms at 1 month of age, and interictal electroencephalogram showed suppression-burst pattern, leading to the diagnosis of OS. She had a heterozygous c.902+5G>A mutation of *STXBP1*, which affects donor splicing of exon 10, resulting in 138-bp insertion of intron 10 sequences in the transcript. The mutant transcript had a premature stop codon, and was degraded by nonsense-mediated mRNA decay in lymphoblastoid cells derived from the patient. High-resolution melting analysis of clinically unaffected parental DNAs suggested that the father was somatic mosaic for the mutation, which was also suggested by sequencing. Cloning of PCR products amplified with the paternal DNA samples extracted from blood, saliva, buccal cells, and nails suggested that 5.3%, 8.7%, 11.9%, and 16.9% of alleles harbored the mutation, respectively. This is a first report of somatic mosaicism of an *STXBP1* mutation, which has implications in genetic counseling of OS.

Conflict of interest

None of the authors has any conflict of interest to disclose.

H Saitsu[a], H Hoshino[b], M Kato[c], K Nishiyama[a], I Okada[a], Y Yoneda[a], Y Tsurusaki[a], H Doi[a], N Miyake[a], M Kubota[b], K Hayasaka[c] and N Matsumoto[a]

[a]Department of Human Genetics, Yokohama City University Graduate School of Medicine, Fukuura 3-9, Kanazawa-ku, Yokohama 236-0004, Japan, [b]Division of Neurology, National Center for Child Health and Development, Okura 2-10-1, Setagaya-ku, Tokyo 157-8535, Japan, and [c]Department of Pediatrics, Yamagata University Faculty of Medicine, Iida-nishi 2-2-2, Yamagata 990-9585, Japan

Key words: HRM analysis – OS – somatic mosaicism – *STXBP1*

Corresponding author: Dr Hirotomo Saitsu, Department of Human Genetics, Yokohama City University Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan.
Tel.: +81-45-787-2606;
fax: +81-45-786-5219;
e-mail: hsaitsu@yokohama-cu.ac.jp

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Ohtahara syndrome (OS), also known as early infantile epileptic encephalopathy with suppression-burst, is one of the most severe and earliest forms of epilepsy (1). It is characterized by early onset of seizures, typically frequent epileptic spasms, seizure intractability, characteristic suppression-burst patterns on electroencephalogram (EEG), and poor outcome with severe psychomotor retardation (2, 3). Brain malformations such as cerebral dysgenesis or hemimegalencephaly are often associated with OS, but cryptogenic or idiopathic OS is found in a subset of OS patients, in whom genetic aberrations might be involved (4). Mutations in *ARX* gene have been found in several male patients with OS (5–8). We have recently found *de novo* mutations in *STXBP1* (encoding syntaxin binding protein 1, also known as MUNC18-1) in individuals with cryptogenic OS (9). A microdeletion involving *STXBP1* and various kinds of point mutations including missense, frameshift, nonsense, and splicing mutations have been found in about one-third of Japanese cases with cryptogenic OS (10). We have showed that both missense mutations and

a splicing mutation result in haploinsufficiency of *STXBP1*: degradation of STXBP1 proteins with missense mutations and nonsense-mediated mRNA decay (NMD) associated with an aberrantly spliced mRNAs (10).

Here we describe a family with an affected daughter with an *STXBP1* mutation and healthy parents. Parental analysis indicates that the father is somatic mosaic for the mutation. Detailed molecular analysis is presented.

Materials and methods

Patient and her parents

The 1-year-old girl is a product of unrelated healthy parents. There is no history of epilepsy in her parents. She was born at term without asphyxia after an uneventful pregnancy. Her physical and neurological findings were normal until vomitting, which was supposed to be a presymptomatic event of seizures, was observed at 25 days of age, and her seizures started at 37 days of age, consisting of brief tonic spasms, occasionally in cluster, followed by vomiting and subtle seizures, such as head extension, upward eye gazing, and vocalization, with increased muscle tone of her extremities for a few seconds. According to suppression-burst pattern on EEG (Fig. 1a,b), she was diagnosed as OS. Brain magnetic resonance imaging (MRI) showed normal brain structure (Fig. 1c–f). Seizures were refractory to antiepileptic drugs, such as high-dose phenobarbital, phenytoin, zonisamide, pyridoxal phosphate, valproic acid, ketogenic diet, and potassium bromide. Injection of adrenocorticotropic hormone (ACTH) was partially effective. She was hypertonic and could not control her head or smile. At 6 months of age, a mild rigospastic quadriplegia was noted. Developmental milestones were profoundly delayed.

DNA samples

Peripheral blood leukocytes from the patient and her parents as well as other tissues from the father were used for this study. Genomic DNA from whole blood, saliva, buccal cells, and nails were isolated using a Wizard Genomic DNA Purification Kit (Promega, Tokyo, Japan), an Oragene DNA kit (DNA Genotek, Ottawa, Canada), an ISOHAIR kit (Nippon Gene, Toyama, Japan), and a Gentra Puregene Buccal Cell Kit (Gentra, Minneapolis, MN), respectively. Experimental protocols were approved by Institutional Review Boards for Ethical Issues at Yokohama City University School of Medicine and Yamagata University Faculty of Medicine. Informed consent was obtained from the patient's parents in agreement with the requirements of Japanese regulations.

Mutation analysis and TA cloning

Mutation screening of *STXBP1* by high-resolution melting (HRM) analysis using RotorGene-6200 HRM (Corbett Life Science, Brisbane, Australia) was performed as previously described (10). Parentage was confirmed by microsatellite analysis (9). For measurement of the ratio of wild-type and mutant alleles, PCR products using paternal DNA as a template were subcloned into pCR4-TOPO vector (Invitrogen, Carlsbad, CA). Cloned fragments were amplified with PCR mixture containing 1 × ExTaq buffer, 0.2 mM each dNTP, 0.5 μM each primer, and 0.375 U Ex TaqHS polymerase (Takara Bio, Ohtsu, Japan). M13 forward (5′-TAAAACGACGGCCAGTGAAT-3′) and M13 reverse (5′-CAGGAAACAGCTATGACCAT GA-3′) primers were used for amplification, and an ex10-F (5′-AGCTGAAGAGGGTTCGAT GA-3′) primer was used for sequencing.

RNA analysis

RNA analysis using lymphoblastoid cells (LCL) was performed essentially as previously described (10). Briefly, after incubation with dimethyl sulfoxide (as vehicle control) or 30 μM cycloheximide (Sigma, Tokyo, Japan) for 4 h, total RNA was extracted using RNeasy Plus Mini Kit (Qiagen, Tokyo, Japan). Two micrograms total RNA was subjected to reverse transcription, and 1 μl cDNA was used for PCR. Primer sequences are ex9-F (5′-CCCTGTGCTCCATGAATTGAC TTT-3′) and ex12-R (5′-CTGAGGCATCTTCTTC AGCATCTGG-3′). Inhibition of NMD was estimated according to the density ratios of lower normal and upper aberrant bands with/without 30-μM cycloheximide treatments in the culture of the patient's LCL. Two separately extracted RNA samples were used for duplicated experiments, respectively. Data were averaged and the standard deviation was calculated. Statistical analyses were performed using the unpaired Student's *t*-test (two-tailed). DNA of each PCR band purified by QIAEXII Gel extraction kit (Qiagen, Tokyo, Japan) was sequenced.

Results

Through the screening for *STXBP1* mutations in individuals with cryptogenic OS, we found a patient harboring heterozygous c.902+5G>A mutation. To examine whether the mutation

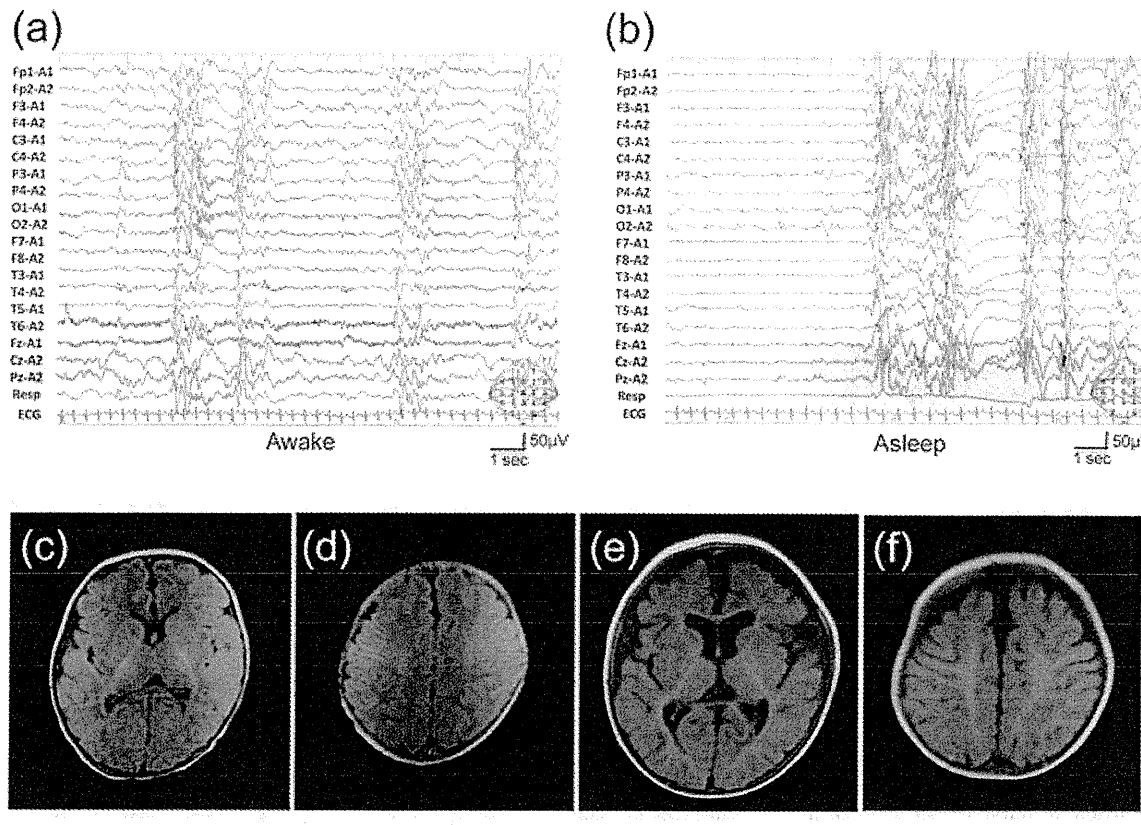

Fig. 1. Interictal electroencephalogram (EEG) (**a**, **b**) and brain magnetic resonance imaging (MRI) (**c**–**f**) of the patient. EEG during both waking (**a**) and sleep (**b**) at 1 month of age showed suppression-burst pattern consisting of low-voltage, almost flat phase and high-voltage paroxysmal activity phase. Brain MRI showed normal findings at 1 month of age (**c**, **d**), and slightly dilated lateral ventricles at 11 months of age (**e**, **f**) because of adrenocorticotropic hormone injection.

could affect donor splicing of exon 10, reverse transcriptase (RT)–PCR designed to amplify exons 9–12 was performed using total RNA extracted from LCL derived from the patient (Fig. 2a). A single band (286 bp), corresponding to the wild-type *STXBP1* allele, was amplified using a cDNA template from a control LCL (Fig. 2b). By contrast, a longer band was detected from the patient's cDNA (Fig. 2b). The longer mutant transcript had a 138-bp insertion of intron 10 sequences (Fig. 2c), producing a premature stop codon at amino acid position 302; therefore, the mutant mRNAs are probably to be degraded by NMD (11, 12). The intensity ratio of the mutant compared to the normal band was increased up to 36.3% after treatment with 30 μM cycloheximide, which inhibits NMD, compared to 13.8% in the untreated condition (Fig. 2d). Thus the mutant transcript suffered from degradation by NMD, which would result in haploinsufficiency of *STXBP1*.

To examine whether the c.902+5G>A mutation occurred *de novo*, the parental DNA extracted from whole blood were analyzed by HRM. Compared with the mother's sample, the patient's sample showed clearly shifted melting curve, indicating that the heterozygous c.902+5G>A mutation could be surely detected (Fig. 3a). Interestingly, the father's sample showed a slightly shifted melting curve, suggesting that the father may harbor the mutation in mosaic state, which was suggested by sequencing (Fig. 3a,b). Similar melting curves and electropherograms were obtained in DNA extracted from saliva, buccal cells, and nails (Fig. 3a,b). We further investigated the mosaicism by counting wild-type G and mutant A alleles after TA cloning of the PCR product. DNA extracted from blood, saliva, buccal cells, and nails suggested that 5.3%, 8.7%, 11.9%, and 16.9% of alleles (i.e. 10.6%, 17.4%, 23.8%, and 33.8% of cells) harbored the mutation, respectively (Fig. 3c).

Discussion

To date, 13 point mutations and one deletion of *STXBP1* have been reported in individuals with OS (9, 10). Thirteen out of fourteen deletion/mutations were confirmed as *de novo*

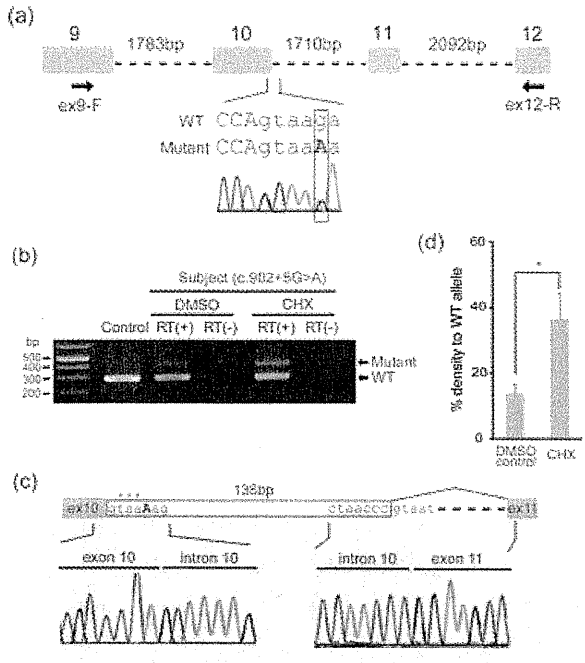

Fig. 2. The c.902+5G>A mutation causing abnormal splicing associated with nonsense-mediated mRNA decay (NMD). **(a)** Schematic representation of the genomic structure from exons 9 to 12 of *STXBP1*. Exons, introns and primers are shown by gray boxes, dashed lines and arrows, respectively. The mutation in intron 10 was colored in red. Sequences of exon and intron are presented in upper and lower cases, respectively. **(b)** Reverse transcriptase (RT) – PCR analysis of the patient with c.902+5G>A and a normal control. Two PCR products were detected from the patient's cDNA: lower was the wild-type (WT) transcript and upper was the mutant. Only a single WT amplicon was detected in a control. The mutant amplicon was significantly increased by 30-μM cycloheximide (CHX) treatment compared to DMSO treatment as a vehicle control. RT (+): with reverse transcriptase, RT (−): without reverse transcriptase as a negative control. **(c)** Sequence of mutant amplicons clearly showed a 138-bp insertion of intron 10 sequences and a premature stop codon (asterisk) in the mutant transcript. **(d)** Quantitative analysis of the NMD inhibition by CHX based on the data shown in **(b)**. *p = 0.00186 by unpaired Student's *t*-test (two-tailed). Averages of duplicated experiments using two distinctive RNA samples, respectively, are shown with error bars (standard deviation).

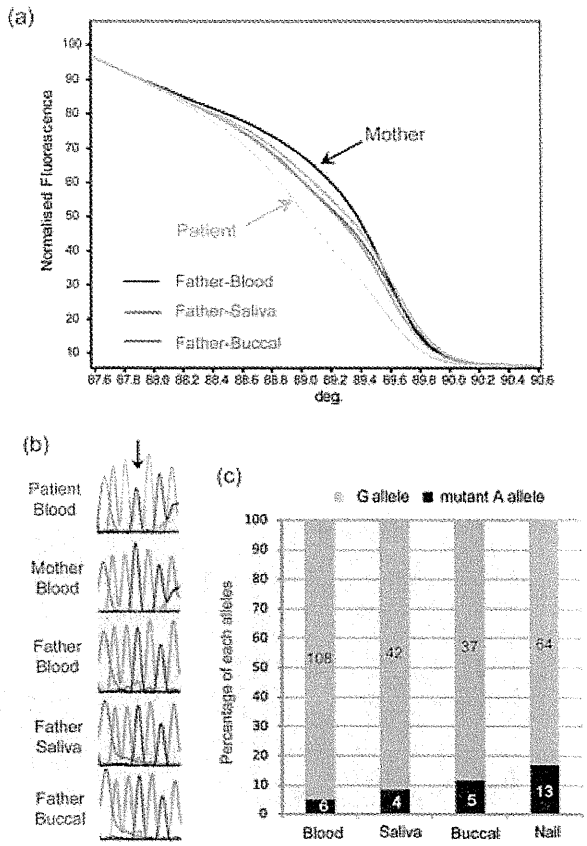

Fig. 3. Paternal somatic mosaicism of the c.902+5G>A mutation. **(a)** Melting curves of PCR products. Compared with the mother's sample (black), the patient's sample (gray) showed largely shifted melting curve. The father's sample from blood (red), saliva (green), and buccal cells (blue) showed slightly, but distinctly shifted melting curves. **(b)** Electropherograms of the c.902+5G>A mutation (arrow) showed mosaicism of the mutation in the father. **(c)** Allele frequencies counted by TA cloning of PCR products and sequencing. DNA extracted from blood, saliva, buccal cells, and nails of the father showed that 5.3%, 8.7%, 11.9%, and 16.9% of alleles harbored the mutant A allele. The numbers of colonies corresponding to each allele are indicated within bars.

events (paternal DNA was unavailable for one remaining mutation). Many OSs are sporadic, probably because of their poor outcome with severe psychomotor retardation; however, some X-linked familial cases have been reported with *ARX* mutations (6, 8). Here we have showed a paternal somatic mosaicism of an *STXBP1* mutation. Although DNA from the semen of the father could not be analyzed in this study, the identical c.902+5G>A mutation found in both the father and the affected daughter indicated that the father should possess the mutation in germ cells as a mosaic state, suggesting recurrence risks. Thus, somatic and germline mosaicism of *STXBP1* mutations should be carefully taken into account especially for genetic counseling of familial OS cases.

We have successfully identified the paternal somatic mosaicism of an *STXBP1* mutation by HRM. DNA from blood indicated that the mosaic ratio is as low as about 5%; therefore, HRM could be very sensitive in detecting low-ratio mosaicism. HRM is a rapid and simple approach to detect heteroduplexes (13). It only requires the addition of a saturating dye before PCR. By HRM analysis of the PCR products, the sensitivity of successful detection of heterozygotes is nearly 100% (13). It should be noted that the sensitivity of HRM to

detect somatic changes or heteroplasmy is much better than that of DNA sequencing (14, 15): HRM could detect the level of somatic mosaicism down to 5–10% (15). However, the ability to detect low percentage heterodeuplex of PCR products may vary among mutations. Although the heterozygous c.902+5G>A mutation showed largely shifted melting curve, we experienced some heterozygous mutations only showing slightly shifted melting curve, in which we may not be able to detect the mosaicism. Therefore, optimization of HRM analysis for each mutation would be recommended especially to examine parental samples.

In conclusion, we firstly described the paternal somatic mosaicism of an *STXBP1* mutation. The percentage of mosaicism was quite low (5–17%), and no minor problems like dexterity, intelligence (cognition), behavior or psychological state were recognized in the father. The information described here was quite useful for future genetic counseling of this family.

Acknowledgements

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ARTICLE

SMOC1 Is Essential for Ocular and Limb Development in Humans and Mice

Ippei Okada,[1,14] Haruka Hamanoue,[1,2,14] Koji Terada,[3] Takaya Tohma,[4] Andre Megarbane,[5] Eliane Chouery,[5] Joelle Abou-Ghoch,[5] Nadine Jalkh,[5] Ozgur Cogulu,[6] Ferda Ozkinay,[6] Kyoji Horie,[7] Junji Takeda,[7,8] Tatsuya Furuichi,[9,10] Shiro Ikegawa,[9] Kiyomi Nishiyama,[1] Satoko Miyatake,[1] Akira Nishimura,[1] Takeshi Mizuguchi,[1,15] Norio Niikawa,[11,12] Fumiki Hirahara,[2] Tadashi Kaname,[13] Koh-ichiro Yoshiura,[12] Yoshinori Tsurusaki,[1] Hiroshi Doi,[1] Noriko Miyake,[1] Takahisa Furukawa,[3] Naomichi Matsumoto,[1,*] and Hirotomo Saitsu[1,*]

Microphthalmia with limb anomalies (MLA) is a rare autosomal-recessive disorder, presenting with anophthalmia or microphthalmia and hand and/or foot malformation. We mapped the MLA locus to 14q24 and successfully identified three homozygous (one nonsense and two splice site) mutations in the SPARC (secreted protein acidic and rich in cysteine)-related modular calcium binding 1 (*SMOC1*) in three families. *Smoc1* is expressed in the developing optic stalk, ventral optic cup, and limbs of mouse embryos. *Smoc1* null mice recapitulated MLA phenotypes, including aplasia or hypoplasia of optic nerves, hypoplastic fibula and bowed tibia, and syndactyly in limbs. A thinned and irregular ganglion cell layer and atrophy of the anteroventral part of the retina were also observed. Soft tissue syndactyly, resulting from inhibited apoptosis, was related to disturbed expression of genes involved in BMP signaling in the interdigital mesenchyme. Our findings indicate that SMOC1/Smoc1 is essential for ocular and limb development in both humans and mice.

Introduction

Microphthalmia with limb anomalies (MLA [MIM 206920]), also known as Waardenburg anophthalmia syndrome or ophthalmoacromelic syndrome, is a rare autosomal-recessive disorder first described by Waardenburg.[1] It is characterized by ocular anomalies ranging from mild microphthalmia to true anophthalmia and by limb anomalies such as oligodactyly, syndactyly, and synostosis of the 4th and 5th metacarpals.[2–4] The genetic cause for MLA has remained unknown.

It is widely known that secreted signaling molecules such as Sonic hedgehog (Shh), wingless-type MMTV integration site family (Wnt), transforming growth factor β (Tgf-β), bone morphogenetic proteins (Bmps), and fibroblast growth factor (Fgf) are involved in the development of many organs and tissues, including the eyes and limbs.[5,6] In particular, mutations in *BMP4* (MIM 112262) have resulted in anophthalmia with systemic manifestations, including polydactyly and/or syndactyly (also known as micropthalmia, syndromic 6, MCOPS6 [MIM 607932]),[7] highlighting importance of BMP signaling in both the developing eye and limb.

SMOC1 (MIM 608488), which encodes SPARC (secreted protein acidic and rich in cysteine)-related modular calcium binding 1, is a member of the SPARC (also known as BM-40) matricellular protein family that modulates cell-matrix interaction by binding to many cell-surface receptors, the extracellular matrix, growth factors, and cytokines.[8,9] SMOCs are extracellular glycoproteins with five domains: an N-terminal follistatin-like (FS) domain, two thyroglobulin-like (TY) domains, a domain unique to SMOC, and an extracellular calcium-binding (EC) domain.[9] SMOC1 is widely expressed in various tissues with localization to basement membranes.[9,10] Although the biological function of SMOC1 remains largely unknown, it has been recently reported that *Xenopus* smoc protein, the ortholog of human SMOC1, acts as a BMP antagonist,[11] suggesting that human SMOC1 can also modulate BMP signaling.

Here, we demonstrate that *SMOC1* mutations cause MLA. We also show that *Smoc1* null mice recapitulated

[1]Department of Human Genetics, Yokohama City University Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan; [2]Department of Obstetrics and Gynecology, Yokohama City University Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan; [3]Department of Developmental Biology, Osaka Bioscience Institute, 6-2-4 Furuedai, Suita, Osaka 565-0874, Japan; [4]Division of Pediatrics, Okinawa Prefectural Nanbu Medical Center & Children's Medical Center, 118-1 Ikyoku, Arakawa, Haebaru, Okinawa 901-1193, Japan; [5]Medical Genetics Unit, St. Joseph University, Beirut 1104-2020, Lebanon; [6]Department of Pediatrics, Ege University Faculty of Medicine, 35100 Bornova-Izmir, Turkey; [7]Department of Social and Environmental Medicine, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan; [8]Center for Advanced Science and Innovation, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan; [9]Laboratory for Bone and Joint Disease, Center for Genomic Medicine, RIKEN, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan; [10]Laboratory Animal Facility, Research Center for Medical Sciences, Jikei University School of Medicine, 3-25-8, Nishi-Shimbashi, Minato-ku, Tokyo 105-8461, Japan; [11]Research Institute of Personalized Health Sciences, Health Sciences University of Hokkaido, Ishikari-Tobetsu, Hokkaido 061-0293, Japan; [12]Department of Human Genetics, Nagasaki University Graduate School of Biomedical Sciences, Sakamoto 1-12-4, Nagasaki 852-8523, Japan; [13]Department of Medical Genetics, University of the Ryukyus Faculty of Medicine, 207 Uehara, Nishihara, Okinawa 903-0215, Japan
[14]These authors contributed equally to this work
[15]Current address: Laboratory of Biochemistry and Molecular Biology, National Cancer Institute, National Institutes of Health, Building 37, Room 6050, Bethesda, MD 20892, USA
*Correspondence: naomat@yokohama-cu.ac.jp (N.M.), hsaitsu@yokohama-cu.ac.jp (H.S.)
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MLA phenotypes, indicating that *SMOC1* plays essential roles in both eye and limb development in humans and mice.

Subjects and Methods

Subjects

A total of four families with one or two cases of MLA were analyzed in this study, including three previously reported families (A, B, and C).[12,13] Family X from Turkey, which has been previously described,[14] was newly recruited to this study. Detailed clinical information of all the patients is available in the literature,[12,14] and phenotypes of patients with confirmed mutations are summarized in Table S1 (available online). A total of five affected and 16 unaffected members from the four families were analyzed in the linkage study. Genomic DNA was obtained from peripheral-blood leukocytes with the use of QuickGene 610-L (Fujifilm, Tokyo, Japan) after informed consent had been given. Experimental protocols were approved by the institutional review board of Yokohama City University School of Medicine.

SNP Genotyping, and Fine Mapping with Short Tandem Repeat Markers

Whole-genome SNP genotyping, with the use of GeneChip Human Mapping 50K Array XbaI (Affymetrix, Santa Clara, CA), and fine mapping of possible candidate regions, with the use of additional microsatellite markers, were performed as previously described.[12,15] The list of primers used for fine mapping is presented in Table S2.

Linkage Analysis

Multipoint linkage analyses using aligned SNPs were performed with ALLEGRO software.[16] Two-point linkage analyses of candidate regions were performed with the LINKAGE package MLINK (FASTLINK software, version 5.1). In each program, an autosomal-recessive model of inheritance with complete penetrance and a disease-allele frequency of 0.001 were applied.

Mutation Analysis of Candidate Genes

All coding exons and exon-intron boundaries of *RAD51L1* (MIM 602948), *ACTN1* (MIM 102575), *ERH* (MIM 601191), *SRSF5* (MIM 600914), *DCAF5* (MIM 603812), *COX16*, *EXD2*, *GALNTL1*, *SLC39A9*, *KIAA0247*, *MED6* (MIM 602984), *TTC9* (MIM 610488), *MAP3K9* (MIM 600136), and *SMOC1* (transcript variant 1, GenBank accession number NM_001034852.1) were analyzed in the probands of families A, C, and X. The transcript variant 2 of *SMOC1* (GenBank accession number NM_022137.4) is 3 bp shorter than the variant 1, leading to an in-frame amino acid deletion at position 431. PCR was cycled 35 times at 94°C for 30 s, at 60°C for 30 s, and at 72°C for 30–90 s in a total volume of 20 μl containing 30 ng genomic DNA as a template, 0.5 μM forward and reverse primers, 200 μM each deoxyribonucleotide triphosphate (dNTP), 1 × ExTaq buffer, and 0.25 U ExTaq (Takara). All primers were designed with Primer3 software. Detailed information of primers is available upon request. PCR products were purified with ExoSAP (USB) and sequenced with BigDye Terminator 3.1 (Applied Biosystems) on a 3100 Genetic Analyzer. Sequences of patients were compared to reference genome sequences in the UCSC Genome Browser (February 2009 assembly) with Seqscape software, version 2.1 (Applied Biosystems).

Animals

Smoc1 mutant mice, created with the use of the *Sleeping Beauty* transposon system, have been previously described.[17] Line PV384 was provided by the RIKEN BioResource Center through the National BioResource Project of MEXT, Japan. Three independent mouse lines (no. 1 to no. 3), each with a single insertion in intron 1 of *Smoc1*, were bred as heterozygotes. Lines 1 and 3 were backcrossed for at least four generations to a C57BL/6J background. Line 2 was maintained with a mixed background of C57BL/6J and ICR. We mainly analyzed line 1, but we confirmed similar phenotypes in lines 2 and 3. Animals were housed in accordance with protocols approved by the Institutional Animal Care and Use Committee at Yokohama City University, School of Medicine. PCR genotyping of mice was performed with the use of genomic DNA from yolk-sac, ear, or tail biopsies. The following primers were used: PV384-WF, 5′-AAAGGCTGGGAATTGTTTG A-3′; PV384-WR, 5′-TGCAGCTGAAACTGTCTCTCC-3′; PV384-MF, 5′-TGTCCTAACTGACTTGCCAAA-3′. The PV384-WF/PV384-WR primers amplified a 441 bp wild-type (WT) product, and the PV384-MF/PV384-WR primers amplified a 218 bp mutant product.

Southern Hybridization

Genomic DNA was extracted from livers or tail biopsies of PV384 heterozygous (*Smoc1*$^{Tp/+}$) mice via standard protocols. The gene-trap insertions were analyzed by Southern hybridization with the use of 10 μg of *Sac*I-, *Nde*I-, BglII-, and *Eco*RI-digested DNA. The probe (451 bp), which hybridized to the internal ribosome entry site (IRES) in the gene-trap vector, was synthesized with the DIG PCR Probe Synthesis Kit (Roche) with the use of the following primers: 5′-CTAACGTTACTGGCCGAAGC-3′ and 5′-CCCAGATCAGATCCCATACAA-3′. Hybridization, washing, and detection of probes were performed according to the manufacturer's protocol. Images were captured with the FluorChem system (Alpha Innotech).

Cloning of Gene-Trap Insertion Sites

After identification of aberrant DNA fragments by Southern hybridization, *Nde*I-, *Sac*I-, and *Eco*RI-digested DNA from PV384 mice was fractionated by electrophoresis, and appropriately sized fragments containing *Ol1* (*other locus 1*), *Ol2*, and *Ol3* were isolated with a QIAEXII Gel Extraction Kit (QIAGEN). The isolated DNA was self-ligated by Ligation High ver.2 (Toyobo), precipitated with ethanol, and dissolved in 20 μl EB buffer (QIAGEN). Inverse PCR was performed in 25 μl reactions, containing 2 μl ligated DNA, 1 × PCR buffer for KOD FX, 0.4 mM each dNTP, 0.5 μM each primer, and 0.5 U KOD FX DNA polymerase (Toyobo). Primers common to *Ol1*, *Ol2*, and *Ol3* were as follows: Inv-F, 5′- AT CGCCAGTTCTGTATGAACGGTCTGGTCTT-3′; Inv-R, 5′-CCCTC TTTACGTGCCAGCCATCTTAGAGATAC-3′. Confirmatory PCR of gene-trap insertion sites for *Ol1*, *Ol2*, and *Ol3* loci was performed with the use of the following primers: *Ol1*-F, 5′-GAGTGGTATTCA TTGGATTCTGCTGAT-3′; *Ol2*-F, 5′-AAATCCAGCTGGCCAACAGA CTAAG-3′; *Ol3*-F, 5′-TTGCCGGGTAGACTCTATCAAGAACCA-3′; TBAL-R, 5′-CTTGTGTCATGCACAAAGTAGATGTCC-3′. Primer sets of *Ol1*-F/TBAL-R, *Ol2*-F/TBAL-R, and *Ol3*-F/TBAL-R could amplify 175 bp, 607 bp, and 767 bp products, respectively. These PCR primer pairs were also used for genotyping of mice harboring a single insertion at the *Smoc1* locus.

Confirmation of Promoter- and Poly(A)-Trapped Transcripts

Whole embryos at embryonic day 10.5 (E10.5) and E11.5 were stored in RNAlater solution (QIAGEN). Total RNA was extracted from WT, *Smoc1*$^{Tp/+}$, and *Smoc1*$^{Tp/Tp}$ embryos with the use of RNeasy Plus Mini (QIAGEN). One microgram total RNA was subjected to reverse transcription with the use of a PrimeScript 1st Strand Synthesis Kit with random hexamers (Takara). A control reaction with no reverse transcriptase was included in each experiment. PCR was performed in 20 μl reactions, containing 1 μl cDNA, 1 × PCR Buffer for KOD FX, 0.4 mM each dNTP, 0.3 μM each primer, and 0.4 U KOD FX (Toyobo). Primers used are listed below: *Smoc1*-F, 5′-GTCCCCACCTCCCCAAGTGCTTTGA-3′; *LacZ*-R, 5′-TGCCAAAAGACGGCAATATGGTGGAAA-3′; *GFP*-F, 5′-TCACATGGTCCTGCTGGAGTTCGTGAC-3′; *Smoc1*-R, 5′-ACACTTGCTCTGGCCAGCATCTTTGCAT-3′. Primer sets of *Smoc1*-F/*Smoc1*-R, *Smoc1*-F/*LacZ*-R, and *GFP*-F/*Smoc1*-R could amplify native *Smoc1* (366 bp), promoter-trapped transcripts (Tp-LacZ, 500 bp) and poly(A)-trapped transcripts (Tp-GFP, 308 bp), respectively. The PCR conditions were 98°C for 10 s, 68°C for 1 min, for 30 cycles. Primers for *ACTB*[18] were used as an internal control. PCR for *ACTB* was cycled 20 times at 94°C for 20 s, 60°C for 20 s, and 72°C for 30 s in a total volume of 10 μl containing 0.5 μl cDNA, 0.4 μM each primer, 0.2 mM each dNTP, 1 × ExTaq buffer, and 0.5 U ExTaq HS (Takara). All PCR products were electrophoresed on 2% agarose gels.

In Situ Hybridization

Embryos were collected between E9.5 and E13.5. Whole-mount in situ hybridization was carried out as previously described.[19,20] Two fragments of *Smoc1* cDNA were obtained as probes by RT-PCR, with the use of total RNA extracted from livers of E16.5 mouse embryos, and subcloned into pCR4-TOPO (Invitrogen). Primer sequences were as follows: probe 1-F, 5′-GTCTGCTCACGCCCCACT-3′; probe 1-R, 5′-CCTGAACCATGTCTGTGGTG-3′; probe P-F, 5′-CAGGAACAGGAAAGGGAAGA-3′; probe P-R, 5′-AAGGGAAAACCACACAGCAC-3′. PCR products were 1023 bp and 1578 bp, corresponding to nucleotide positions 275–1297 and 1849–3426 of the mouse *Smoc1* cDNA (GenBank accession number NM_001146217.1), respectively. The cDNA fragment amplified with probe P-F and probe P-R primers was identical to the probe used in a previous report.[21] Digoxigenin-labeled sense and antisense riboprobes were synthesized with the use of a digoxigenin RNA labeling kit (Roche). These two different antisense probes demonstrated identical staining patterns, and the control sense probes showed no staining. The expression pattern was confirmed with more than three embryos. In addition, the following probes were used: *Bmp2* (gift from Y. Takahashi),[22] *Sox9* (gift from A. Yamada),[22] *Bmp7* (gift from E.J. Robertson), and *Msx2* (gift from Dr. R.E. Maxson, Jr). The numbers of embryos examined were as follows (numerical quantity for WT, *Smoc1*$^{Tp/+}$, and *Smoc1*$^{Tp/Tp}$, respectively, shown in parentheses): *Msx2* (2, 1, 3) at E11.5; *Bmp2* (3, 0, 3), *Bmp7* (3, 0, 3), *Msx2* (3, 0, 3), and *Sox9* (2, 1, 3) at E12.5; *Bmp2* (1, 2, 3), *Bmp7* (2, 1, 3), *Msx2* (1, 2, 3), and *Sox9* (1, 3, 4) at E13.5. Stained embryos were cleared in glycerol to enable images to be produced with a VHX-1000 digital microscope (Keyence).

Histology

Heads of embryos and newborns were fixed overnight in 4% paraformaldehyde in PBS at 4°C. These embryos were then washed in PBS. Frozen samples were serially sectioned at 16 μm (E14.5) and 20 μm (P0). The numbers of eyes examined (WT, *Smoc1*$^{Tp/+}$, *Smoc1*$^{Tp/Tp}$) were as follows: coronally sectioned at E14.5 (8, 10, 12), coronally sectioned at P0 (8, 10, 6), horizontally sectioned at P0 (2, 2, 4). For evaluation of ventral atrophy of the retina, only the coronally sectioned eyes were used. TB staining was performed according to standard protocols. Forelimbs of mice were fixed in 4% paraformaldehyde in PBS, decalcified in 10% EDTA, and embedded in paraffin. Forelimbs were serially sectioned at 4 μm and stained with hematoxylin and eosin.

Evaluation of Optic Nerve Diameter

The palatine and orbital bones were carefully removed to expose the optic chiasm and optic nerve. During the dissection process, 4% paraformaldehyde in PBS was frequently applied onto the gaps between the bone and optic nerve. Xylene cyanol was applied to enhance the outline of optic nerves at poastnatal day 0 (P0). Photographs of optic nerves were taken with a VHX-1000 digital microscope, and the diameter was measured for right and left optic nerves with the bundled software included with the VHX-1000 instrument.

Skeletal Staining

For skeletal preparations, mice were fixed in 99.5% ethanol after removal of the skin and viscera. Cartilage tissues were stained with 0.015% alcian blue and 20% acetic acid in 75% ethanol for three days at 37°C. After dehydration with 99.5% ethanol for three days, bones were stained with 0.002% alizarin red in 1% KOH. Then skeletons were cleared in 1% KOH for several weeks. For P14 mice, soft tissues were dissolved in 2% KOH before alizarin red staining.

Nile Blue Staining

For the study of apoptosis of hindlimbs at E13.5 and E14.5, Nile blue (NB) staining was performed on the basis of a previously described protocol,[23] except that staining was performed at 37°C (not room temperature). Apoptosis was determined by NB-stained (deceased) cells. After rinsing in Tyrode solution, hindlimbs of control (WT and heterozygous littermates) and homozygous mice were evaluated. Photographs of dorsal aspects were taken with a VHX-1000 digital microscope. Experiments were repeated three times, and reproducible representative results are presented.

Statistical Analysis

Statistical analyses were performed with the use of non-repeated-measures ANOVA followed by Dunnett's post hoc test. The results are given as mean ± standard deviation, and the threshold p value for statistical significance was 0.01.

Results

Identification of Homozygous *SMOC1* Mutations

We have previously mapped the MLA locus to a 422 kb region at 10p11.23 by analyzing three families (one Japanese family [A] and two Lebanese families [B and C]). This region contained only one gene, *MPP7*, in which no mutations were found.[12] After a new Turkish family (X) was added to the analysis, the MLA locus was again searched by homozygosity mapping to the consanguineous families (X, B, and C) and haplotype mapping to family A for detection of compound-heterozygous mutations; however, we could not detect any common regions

among the four families. We then focused on identifying common regions in any three of the four families to allow for locus heterogeneity (Table S3).

A locus at 14q24.1-q24.2, which showed the highest LOD score (3.936) among the candidate regions larger than 2.0 Mb, was highlighted among families A, C, and X. This locus was analyzed with the use of additional microsatellite markers, and a 3.0 Mb region containing 24 genes was identified (Figures 1A and 1B). A total of 14 genes were sequenced, and homozygous mutations were found in *SMOC1*: c.718C>T (p.Gln240X) in family A, c.664+1G>A in family C, and c.378+1G>A in family X (Figures 1C and 1D). All of these homozygous mutations were cosegregated with the disease phenotype, and the parents of the individuals with these mutations were heterozygous carriers (Figure 1C). We could not find any mutations in *SMOC1* in family B, in which MLA is unlinked to the 14q24.1-q24.2 locus. Interestingly, in family A haplotypes of paternal and maternal alleles, each having the same mutation, are completely different (data not shown), suggesting that the same mutation may have occurred in separate events. The c.718C>T mutation was not detected in 289 healthy Japanese controls, including 100 Okinawa islanders. The other two mutations were not detected in ethnically matched controls (54 Lebanese and 99 Turkish subjects, respectively), nor in 289 Japanese controls. The two splice-donor-site mutations (c.664+1G>A and c.378+1G>A) are predicted to abolish a donor site, as predicted by ESE-finder, NetGene2, HSF2.4.1, SpliceView, and BDGP analysis (Table S4). Thus, the three mutations are likely to lead to a loss of functional SMOC1.

Smoc1 Expression in the Developing Eye and Limb in Mice

For the examination of *Smoc1* expression in the developing eye and limb, whole-mount in situ hybridization of mouse embryos was performed. *Smoc1* was expressed in the forebrain, midbrain, hindbrain, pharyngeal arch, somites, and forelimb buds at E9.5 (Figure 2A). At E10.5, *Smoc1* expression was observed in the optic stalk (Figure 2B), and at E11.5, expression was localized to the closure site of the optic cup (Figure 2C). Expression of *Smoc1* in developing limbs between E10.5 and E11.5 was observed in both dorsal and ventral regions, with a broader pattern of expression in dorsal regions, but expression was not detected in the most anterior, posterior, and distal parts of limb buds (Figures 2D and 2E). Expression coinciding with chondrogenic condensation was observed at E12.5 (Figure 2F), and expression then became restricted to future synovial joint regions at E13.5 (Figure 2G). This dynamic expression suggests that *Smoc1* plays a critical role in ocular and limb development.

Ocular and Limb Anomalies in *Smoc1* Null Mice

To investigate the pathological basis of MLA due to the loss of *SMOC1* function, we obtained *Smoc1* mutant mice, PV384.[17] PV384 mice possess gene-trap insertions in the *Smoc1* locus and in three other loci. After PV384 mice were bred with C57BL/6J or ICR mice, we obtained three independent lines (no. 1 to no. 3), each with a sole insertion in intron 1 of *Smoc1* (Figure S1). We mainly analyzed line 1, but we confirmed similar phenotypes in lines 2 and 3. Heterozygous mutant mice ($Smoc1^{Tp/+}$) were healthy and fertile. Homozygous mice ($Smoc1^{Tp/Tp}$) were null mutants, as they showed no native transcript of *Smoc1* (Figure S1E). Homozygous mice were viable at P0; however, they did not survive beyond the first 3 wks of life (Figure 3B). Their growth was retarded in comparison to WT and heterozygous littermates at P0 and P14 (Figures 3A and 3C). Developmental defects in eyes and optic nerves were evident at E14.5. Homozygous mice had relatively small eyes, and histological examinations revealed aplasia or hypoplasia of optic nerves (in 10 of 12 optic nerves), atrophy of the anteroventral part of the retina (in 11 of 12 eyes), and extension of the retinal pigmented epithelium (RPE) to the optic nerve (in 10 of 12 eyes) (Figures 3D–3I). These abnormalities were also observed at P0 (aplasia or hypoplasia of optic nerves [in 7 of 10 optic nerves], retinal atrophy [in 6 of 6 eyes], and RPE extension [in 3 of 6 eyes with identifiable optic nerves]) (Figures 3J–3M). WT or heterozygous littermates did not show any such abnormalities, except that a few eyes of heterozygous mice showed extension of the RPE at E14.5, but not at P0 (in 2 of 10 and 0 of 12 eyes, respectively). Toluidine blue (TB) staining showed ganglion cell layers that were thinned and irregular to varying degrees in homozygous mice, suggesting a reduced number of retinal ganglion cells (Figures 3J–3K′). Thus, *Smoc1* is required for axon sprouting, elongation, or maintenance of retinal ganglion cells.[24] Hypoplasia of optic nerves was further quantitatively confirmed by macroscopic examination: the average diameter of optic nerves of homozygous mice was significantly smaller than that of WT and heterozygous littermates at P0 and P14 (Figures 3L–3Q). These data clearly demonstrate that loss of *Smoc1* in mice affects development of the body, retina, and optic nerves, in a manner similar to that seen in MLA patients.[3,4]

Newborn homozygous mice could be readily identified by their hindlimb syndactyly and pes valgus, whereas no abnormalities were observed in WT and heterozygous pups (Figure 4 and Table 1). Interestingly, the severity of syndactyly varied between mouse lines: line 1 exclusively showed soft tissue syndactyly, whereas line 2 frequently showed four digits (Figures 4F and 4J). Skeletal preparations with alcian blue and alizarin red revealed that the foot with four digits had four phalanx and five metatarsals with fusion to each other (Figure 4K). Thus the *Smoc1* null mutation resulted in a spectrum of phenotypes, from soft tissue syndactyly to four fused digits, probably due to different genetic backgrounds. Bowed tibiae and hypoplastic fibulae were also consistently observed in homozygous mice (Figures 4H and 4L). The articulation between