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Severe mandibuloacral dysplasia caused by novel compound heterozygous *ZMPSTE24* mutations in two Japanese siblings

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Abstract

Mandibuloacral dysplasia (MAD) is a rare autosomal recessive progeroid syndrome, characterized by mandibular hypoplasia, acroosteolysis affecting distal phalanges and clavicles, delayed closure of the cranial sutures, atrophic skin, and lipodystrophy. Recently, mutations in lamin A/C (*LMNA*) and zinc metalloprotease (*ZMPSTE24*), involved in post-translational processing of prelamin A to mature lamin A, have been identified in MAD kindreds. We now report novel compound heterozygous mutations in exon 1 (c.121C>T; p.Q41X) and exon 6 (c.743C>T; p.P248L) in *ZMPSTE24* in two Japanese sisters, 7- and 3-year old, with severe MAD and characteristic facies and atrophic skin. The older sister had lipodystrophy affecting the chest and thighs but sparing abdomen. Their parents and a brother, who were healthy, had heterozygous mutations. The missense mutation, P248L, was not found in 100 normal subjects of Japanese origin. The mutant Q41X was inactive in a yeast halo assay; however, the mutant P248L retained near normal *ZMPSTE24* activity. Immunoblots demonstrated accumulation of prelamin A in the patients' cell lysates from lymphoblasts. The lymphoblasts from the patients also revealed less intense staining for lamin A/C on immunofluorescence. We conclude that *ZMPSTE24* deficiency results in accumulation of farnesylated prelamin A, which may be responsible for cellular toxicity and the MAD phenotype.

Keywords

lamin A; lipodystrophy; mandibuloacral dysplasia; progeroid syndrome; *ZMPSTE24*

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Mandibuloacral dysplasia (MAD) is a rare autosomal recessive, heterogeneous progeroid syndrome. Prominent clinical features include craniofacial anomalies such as mandibular hypoplasia, dental overcrowding, bird-like facies with prominent eyes, and thin beaked nose (1,2). Skeletal anomalies include delayed closure of the cranial sutures, as well as progressive osteolysis of the terminal phalanges and clavicles. Skin anomalies consist of atrophy, mottled hyperpigmentation, and lipodystrophy. Stiff joints and post-natal growth retardation have been reported in many cases. The patients have normal intelligence. Most of the symptoms first appear around the age of 4 years in many reported cases, and these clinical manifestations are progressive. Geographically, many cases were reported from Italy, suggesting the existence of a founder effect (3,4); however, several cases outside Italy have also been reported (5–8).

Two distinct patterns of lipodystrophy have been reported in patients with MAD: type A (OMIM 248370) and type B (OMIM 608612) (5). Type A lipodystrophy is characterized by marked loss of subcutaneous (SC) fat from the extremities with normal or slight excess in the neck and truncal regions. Patients with MAD and type A lipodystrophy have been reported to harbor mutations in the *LMNA* gene (OMIM 150330) on chromosome 1q21, which encodes two nuclear envelope proteins, lamin A and C (6–10). Type B lipodystrophy is characterized by generalized loss of SC fat involving the face, trunk, and extremities. Patients with MAD and type B lipodystrophy have been reported to carry compound heterozygous mutations in the gene encoding an endoprotease, zinc metalloproteinase (*ZMPSTE24*) (OMIM 606480), on chromosome 1p34 (11). *ZMPSTE24* is involved in posttranslational processing of prelamin A to mature lamin A (12); thus, accumulation of prelamin A is proposed to be responsible for the phenotype.

Mutations in *LMNA* gene are also associated with other progeroid syndromes such as Hutchinson–Gilford progeria syndrome (HGPS), restrictive dermopathy (RD), progeria-associated arthropathy, and atypical progeroid syndrome (also referred to as atypical Werner's syndrome) (13–16). Mutations in *ZMPSTE24* gene are also associated with RD (17,18). There are many overlapping features among patients with MAD, HGPS, RD and atypical progeroid syndrome, although each presents with some unique phenotypic features. So far, 21 MAD patients have been reported to have *LMNA* mutations (6–10), but only 3 MAD patients have been reported with *ZMPSTE24* mutations (11,19,20). Here, we report two Japanese siblings with MAD resulting from novel compound heterozygous mutations in *ZMPSTE24*.

Materials and methods

The studies were approved by the Institutional Review Boards of the Osaka University Hospital and the University of Texas Southwestern Medical Center at Dallas. Each participant or their legal guardian gave a written informed consent for all studies and photographs.

Mutational analysis of the *LMNA* and *ZMPSTE24* genes

Genomic DNA was isolated from the subjects' peripheral whole blood cells using Dr. GenTLE (Takara Bio, Otsu, Shiga, Japan) according to the manufacturer's instructions. Direct sequencing of the entire coding region and the flanking exon–intron boundaries of the *LMNA* and *ZMPSTE24* was first conducted in the proband, and the mutations were later confirmed in the younger sister. The primer sets that amplify each exon of *LMNA* and *ZMPSTE24* genes were designed from published sequence information and exons were amplified by polymerase chain reaction (PCR) as previously described (11,17,21,22). *ZMPSTE24* coding sequence is available at <http://genome.ucsc.edu/>. For segregation analyses, only selected exons were sequenced in the family members. The protein sequences of *ZMPSTE24* in various species were obtained from the website of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/Genbank>).

Mutant constructs

The generation of yeast expression vector containing the human *ZMPSTE24* gene has been described (11,19). The mutations Q41X and P248L were generated using the expression vector, pRS416GPD-hZMPSTE24, as a template using QuickChange Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instruction. The mutation Q41X was generated with primer pairs 5'-CCTAGCACAGCGGTAGAGAAGGATATAT-3' (sense) and 5'-ATATATCCTTCTCTACCGCTGTGCTAGG-3' (antisense) and mutation P248L was generated with primer pairs 5'-GAGTATTGACTTTCTTTTGACGAAGG-3' and 5'-CCTTCGTCAAAGAAAGTCAATACTCTTTGCC-3'. The resulting yeast expression vectors were again sequenced to ensure that no PCR errors had occurred.

Growth arrest pheromone diffusion (halo) assay

To determine if the *ZMPSTE24* mutation is active in complementing the yeast STE24 (22, 24), the yeast expression plasmids pRS416GPD-hZMPSTE24-Q41X and pRS416GPD-hZMPSTE24-P248L were transformed into yeast strain SM3614 (*MATa ste24Δrce1Δ*) (a kind gift of Dr S. Michaelis) and grown on selective medium. Liquid cultures were concentrated, and 1 μl aliquots of serial dilutions was then spotted on to a lawn of mating yeast (strain XBH8-2C, *MATα*) on yeast peptone dextrose medium with 0.043% Triton X-100 (24,25), then allowed to grow for 2 days at 30°C. The production of mature a-factor is proportional to the zone of growth inhibition (halo) formed.

Cell culture

Lymphocytes were isolated from the MAD patients (patients 1 and 2) and a healthy volunteer (control) using Ficoll-Paque PLUS (GE Healthcare Bio-Science, Piscataway, NJ) and were immortalized by infecting with Epstein-Barr virus. The cells were cultured in Roswell Park Memorial Institute 1640 medium (Nikken Bio Medical Laboratory, Kyoto, Japan) supplemented with 20% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin and maintained at 37°C and 5% CO₂.

Immunoblotting

Henrietta-Lacks' cervical cancer cells (HeLa) and lymphoblast cell extracts were prepared by harvesting cells in lysis buffer consisting of 1% sodium dodecyl sulfate (SDS), 20 mM NaF and protease inhibitors cocktail (Roche, Indianapolis, IN). The cell lysates were centrifuged at 13,000 g for 15 min at room temperature, and protein concentrations were determined using Bio-Rad DC protein assay (Bio-Rad, Hercules, CA). Total proteins (50 μg) were denatured by heating the samples at 100°C for 10 min and resolved on 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Resolved proteins were transferred onto polyvinylidene fluoride membranes (Immobilon; Millipore, Billerica, MA) and blocked with 5% fat-free milk overnight at 4°C. The blots were probed with 1:5000 diluted goat anti-lamin A/C (N-18; Santa Cruz Biotechnology Inc., Santa Cruz, CA). Proteins were detected with horseradish peroxidase-conjugated secondary antibody (1:2500 dilution) and ECL Plus chemiluminescence system (Amersham Pharmacia, Buckinghamshire, UK). The same blot was stripped using the Restore stripping buffer (Pierce, Rockford, IL) according to the manufacturer's protocol, reprobed with 1:1000 diluted anti-actin antibody (catalog number A 2066; Sigma, St. Louis, MO), and detected with goat anti-rabbit immunoglobulin G (IgG) conjugated with horseradish peroxidase at a dilution of 1:2500.

Immunofluorescence microscopy

Approximately 100,000 lymphoblast cells were seeded onto coverslips coated with poly-L-lysine (Sigma) placed in a six-well plate. Cells were allowed to adhere to the coverslip for 1 h and were then fixed with methanol (−20°C), permeabilized with 0.1% Triton X-100, and

blocked with 5% fetal calf serum and 0.03% bovine serum albumin in phosphate-buffered saline. The permeabilized cells were incubated with an antilamin A/C antibody at 1:100 dilution (H-110; Santa Cruz Biotechnology Inc., Santa Cruz, CA) followed by Alexa Fluor 488, goat anti-rabbit IgG antibody at 1:100 dilution (Molecular Probes/Invitrogen, Carlsbad, CA). Primary and secondary antibodies were incubated for 1 h each at 37°C. The slides then were incubated with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI at 1 µg/ml) for 1 min and mounted with Aqua Poly/Mount (Polysciences Inc., Warrington, PA). Cell imaging was performed by Zeiss Axiovert 100M at similar exposure times. The digital images were prepared using IMAGEJ.

Magnetic resonance imaging technique

Magnetic resonance imaging (MRI) was performed using a 1.5-Tesla imaging device (GE Health Care, Milwaukee, WI). The body was surveyed employing contiguous, axial 5-mm slices, a T1-weighted spin echo sequence [633/8, TR (repetition time)/TE (echo time)], a eight-channel body array coil, 256 × 192 matrix, 0.9 × 0.9 × 5 mm voxel, and one excitation. Fat was easily identified as areas of relatively high signal intensity on images.

Results

Patient 1 (MAD 3300.3)

This 7-year-old female was the first child of healthy and unrelated parents of Japanese origin. She was born at 37 weeks of gestation, with a length of 40 cm (−3.6 SD), a weight of 2230 g (−1.1 SD) and an occipitofrontal circumference (OFC) of 34 cm (+0.7 SD). She had a mild feeding problem and poor weight gain in the infantile period (Fig. 1). Atrophic skin, mandibular hypoplasia and prominent eyes were noted at birth. The right clavicle was accidentally fractured at the age of 1 month. She had a characteristic facial appearance, including mandibular hypoplasia, prominent eyes, and pointed nose. The skin was atrophic, shiny and taut with mottled hyperpigmentation. A contracture developed in the fifth fingers from age of 1 month, and hypoplasia of terminal phalanges was noticed at the age of 1 year. Radiographs of the head showed underdevelopment of the skull and wormian bones at the age of 3 months. Acroosteolysis was not obvious at 3 months but was clearly noticeable at the age of 15 months. These physical and radiological features led to the diagnosis of MAD.

At 1 year of age, her first tooth appeared. Her mental development was normal; however, motor milestones were mildly delayed due to stiffness in multiple joints. She could walk at 16 months. She developed crowding of the teeth, leading to reconstructive surgery and extractions of six mandibular teeth at the age of 5 years.

At the age of 7 years and 2 months, her height was 99.9 cm (−4.0 SD) and weight was 12.9 kg (−2.5 SD). Her height gain was further diminished due to kyphosis and contractures of both knees. The hair was sparse and thin with no alopecia or premature graying (Fig. 1a,b). The lambdoidal suture in the skull was not closed (Fig. 1c,d). The mouth and oral cavity were very small with poorly, inwardly, and irregularly implanted teeth and a high-arched palate. The skin on the trunk was thin with clearly visible superficial veins underneath, and healing of small wounds was delayed. SC fat appeared reduced with the exception of the cheeks, arms and thigh, resulting in muscular appearance. She manifested narrow and drooping shoulders and bilateral flexion contractures of the elbows, hips and knees. She had short distal phalanges, broad and hypoplastic nails, contracted fifth fingers, and round fingertips (Fig. 1e,f). Radiographic studies of the hands showed clinodactyly of the fifth finger, osteolysis of the distal phalanges and delayed osseous maturation (Fig. 1f). Skull radiographs revealed mandibular hypoplasia, delayed ossification, and multiple wormian bones in the lambdoidal suture areas in the skull (Fig. 1c,d). She also had hypoplasia of the lateral ends of the clavicles. Osteolytic change of

right femoral head was also demonstrated (Fig. 1g). Her bone age was 6.1 years, and there was no apparent abnormalities in the growth plates. Evaluation by dual-energy X-ray absorptiometry (DXA) (Discovery QDR-1000; Hologic, Bedford, MA) revealed that bone mineral density of the lumbar spine (L2–L4) was 0.455 g/cm^2 , which was lower than that of other girls aged 7 years ($0.627 \pm 0.06 \text{ g/cm}^2$) (26). DXA scan estimated her total body fat to be 18.1%. Regional fat in the arms, legs, and truncal region was 20.9%, 23.0%, and 14.9%, respectively.

The patient had normal hematological and serum chemistry including serum glucose, insulin, and lipoproteins and had a normal female karyotype. Her serum triglycerides were 25 mg/dl, total cholesterol was 209 mg/dl and high-density lipoprotein cholesterol was 54 mg/dl. Urinalysis showed microhematuria. Ultrasonography of kidneys revealed normal size. Serum levels of follicle-stimulating hormone, luteinizing hormone, thyroid-stimulating hormone (TSH), thyroxine, and estradiol were normal and insulin-like growth-factor-1 (IGF-1) was low (90 ng/ml, normal value: 91–344 ng/ml) but insulin-like growth factor binding protein-3 (IGFBP-3) was normal (3.54 $\mu\text{g/ml}$, normal value: 1.76–4.03 $\mu\text{g/ml}$). As compared with a normal 7-year-old healthy Japanese girl, the patient's MRI revealed a slight reduction in SC fat in the chest as well as in the anterior and posterolateral gluteal region and the thighs; however, the SC fat in the arms and abdomen was within the normal range (Fig. 2).

Patient 2 (MAD 3300.5)

This 3-year and 5 months-old younger sister of Patient 1 was born at 37 weeks, with a length of 40 cm (–3.6 SD), a weight of 2130 g (–1.4 SD) and an OFC of 32 cm (–0.5 SD). Atrophic skin, unusual craniofacial appearance and rigidity of the joints were noted at birth. Characteristic facial appearance and radiographs similar to those of her sister prompted a diagnosis of MAD. Her bone age was 2.6 years, and there were no growth plate abnormalities noted on radiographs. She had feeding difficulty, respiratory distress, and poor weight gain in infancy. At the age of 3 years and 4 months, her height was 84.0 cm (–3.1 SD) and weight was 7.8 kg (–3.5 SD). Her physical findings were less severe than her sister. Her SC fat was scarce but mildly spared on the cheeks, arms and thighs. Laboratory data revealed normal hematological and serum chemistry values. Her serum triglycerides were 76 mg/dl, total cholesterol was 180 mg/dl and high-density lipoprotein cholesterol was 52 mg/dl. Urinalysis showed microhematuria. Ultrasonography of kidneys was normal. Her free thyroxine, free triiodothyronine, IGF-1 and IGFBP-3 were all normal, except for a slightly elevated TSH of 5.53 $\mu\text{U/ml}$.

Analysis of *LMNA* and *ZMPSTE24* gene

In the affected subjects from this pedigree, no mutations were demonstrated in *LMNA* gene; however, we found novel compound heterozygous mutations in *ZMPSTE24* gene (Fig. 3). In exon 1, the heterozygous c.121C>T substitution resulted in p.Q41X, and in exon 6, the heterozygous c.743C>T substitution caused p.P248L (Fig. 3 a,b). A synonymous heterozygous single nucleotide polymorphism in exon 6 (rs 2076697, c.651T>C; p.D217D) was noted in all family members except in the father. Each mutation segregated in Mendelian fashion, and the heterozygous carriers, parents and a brother had normal phenotype. These mutations were not detected in 200 chromosomes from unrelated subjects of Japanese ancestry. Partial alignment of *ZMPSTE24* sequences from various species showed that the P248 is well conserved, indicating the important role of the residue in function of the enzyme (Fig. 3c). In addition, the flanking residues 'F' and 'L' were also conserved, suggesting that the tripeptide FPL may be important for the enzymatic activity.

Functional analysis

Halo assay—Human ZMPSTE24 expressed in yeast also processes a-factor and complement the mating defect of the haploid *MAT α* yeast strain SM3614 lacking the *STE24* and *RCE1* genes (27,28). The secreted mature a-factor diffuses into the medium and arrests the growth of the *MAT α* strain, leaving a ‘halo’ surrounding the *MAT α* strain. The size of the halo is roughly proportional to the production of the mature a-factor; however, modest reductions in activity may produce a normal-sized halo as a-factor is normally secreted in excess. The *ste24 Δ* *rce1 Δ* yeast when transfected with an empty vector did not produce a zone of growth inhibition (halo). Expression of human ZMPSTE24 (wild type) produced a halo surrounding the yeast colony (Fig. 4a). The ZMPSTE24, Q41X mutation, was inactive in this assay; however, the mutant P248L in our assay appears to produce a halo of comparable size as the wild-type ZMPSTE24 (Fig. 4a), even when examined at reduced yeast concentration after dilution (data not shown).

Defects in prelamin A processing—Immunoblots on the lymphoblast cell lysates using anti-lamin A/C antibody showed that control cells contained lamins A and C, while the cells from patients 1 and 2 exhibited an additional higher molecular weight band (top panel) corresponding to prelamin A (Fig. 5a). However, the cell lysates from the parents’ lymphoblasts did not reveal any prelamin A accumulation (Fig. 5b).

Immunostaining of lymphoblast nuclei—Immunofluorescence staining for lamin A appeared to be less intense in both the patients compared with the staining in control lymphoblasts (Fig. 6). The cells from patient 2 appeared to have reduced staining compared with those from patient 1. There was, however, no difference in the DAPI staining of the nuclei between the patients and the control.

Discussion

We report that our patients had two novel heterozygous mutations in the *ZMPSTE24* gene, one of them being a null mutation, Q41X, and the other a missense mutation, P248L. Previously, compound heterozygous *ZMPSTE24* mutation F361fsX379 was found with N265S mutation in two patients, and with W340R mutation in one patient presenting with MAD phenotype (11,19,20). Another patient with an overlapping phenotype of HGPS and MAD had a homozygous V402SfsX403 *ZMPSTE24* mutation (29). This patient also harbored a heterozygous *LMNA* R654X mutation, which was predicted to have ameliorated the phenotype somewhat due to inability of R645X mutant lamin A to be farnesylated (29). On the other hand, all patients with RD associated with *ZMPSTE24* mutations have had homozygous null F361fsX379 mutation or have been compound heterozygotes with an F361fsX379 mutation in association with other ostensibly null mutations such as Q417X, P99fsX38 or L91-L209del mutations (17).

The residue proline 248 lies adjacent to the sixth membrane-spanning domain of ZMPSTE24, and mutation P248L was not detected in the 100 unrelated subjects of Japanese ancestry. Additionally, the proline 248 is well conserved across all the species examined, which supports that this mutation is a disease-causing mutation. The results of the yeast halo assay demonstrated that the ZMPSTE24 mutation, Q41X, was totally inactive; however, the mutant P248L was nearly as active as the wild-type ZMPSTE24. Previously, two heterozygous mutations, N265S and W340R, were shown to have slightly reduced ZMPSTE24 enzymatic activity compared with the wild-type construct in the same yeast halo assay. Nonetheless, documentation of prelamin A accumulation in the lysates from the patients’ lymphocytes clearly suggests ZMPSTE24 deficiency. These results suggest that haploinsufficiency combined with even a minor loss of enzyme activity in the remaining allele may be sufficient

to give rise to clinical symptoms of MAD. Because the halo assay is not very sensitive, it is likely that the mutant P248L may have lost 20–25% activity compared with the wild type, which may not be observed in this assay. Furthermore, the structural requirements of ZMPSTE24 for yeast a-factor processing appear to be less stringent than those for prelamin A cleavage. Other sensitive assays to measure ZMPSTE24 activity *in vitro* will clarify the molecular pathogenesis of these cases. Immunostaining of lymphoblasts with lamin A/C antibodies shows slightly reduced staining in the affected patients when compared with the unrelated control lymphoblasts. However, the nuclear morphology remains unaltered. The significance of this observation requires further confirmation in the dermal fibroblasts from these patients.

Our patients' phenotype is more severe than that of MAD patients with *LMNA* mutations, who usually have normal development during the first 4 years of life. Our patients developed skeletal abnormalities, skin atrophy and joint contractures earlier than age 2 years (1). Our patients, who presented with characteristic facies and atrophic skin at birth, represented severe expressions of MAD in early childhood. Skin atrophy and joint rigidity were more severe in the elder sister who also developed osteolysis of the right femoral head.

MAD patients with *LMNA* mutations have been reported to have lipodystrophy of the extremities with sparing of the head, neck and trunk. Previously, two MAD patients with *ZMPSTE24* mutations were reported to have generalized lipodystrophy on the basis of physical examination, but systematic evaluation of their body fat distribution using anthropometric measurements, DXA or MRI had not been conducted (11,20). The third patient with severe progeroid and MAD phenotype due to *ZMPSTE24* mutations also died prematurely at the age of 2 years and 9 months due to respiratory failure. She was reported to have prominent calf muscles due to lipodystrophy (19). Given the lack of objective data related to lipodystrophy in MAD patients with *ZMPSTE24* mutations, we attempted to characterize the lipodystrophy in our patients. Phenotypically, SC fat was nearly absent in patient 1 with the exception of the cheeks, arms and thigh, and this disproportionately distributed adipose tissue was also demonstrated by the DXA scan. MRI studies revealed that patient 1 had slight reduction of SC fat on the anterior and lateral regions of the thigh and gluteal region and trunk SC fat on the extremities, but SC fat in the abdomen was normal. Unfortunately, in our patients, due to tight skin, it was hard to evaluate skinfold thickness. However, our patients may be too young, at the age of 3 and 7 years, to show the full phenotype of lipodystrophy. In patients with MAD due to *LMNA* mutations, the onset of lipodystrophy occurs at puberty, and it is possible that our patients may also lose more SC fat during puberty. Thus, further SC fat loss in these two patients will illuminate the natural history of lipodystrophy in MAD, type B.

Previously, two MAD patients with *ZMPSTE24* mutations were reported to have SC calcified nodules in the phalanges and developed focal segmental glomerulosclerosis, resulting in end-stage renal disease (20). Our patients have not developed any SC nodules in the phalanges. Even though both of them have normal serum creatinine values, both had microhematuria suggestive of renal involvement.

At present, our patients have normal glucose tolerance and lipid profiles. Previously, MAD patients with lipodystrophy and *LMNA* mutations have been reported to have insulin resistance, mild glucose intolerance, and hyperlipidemia (30). Recently, Simha et al. reported four MAD patients with partial lipodystrophy (6), who had normal glucose tolerance but had fasting and post-prandial hyper-insulinemia. Elevated serum triglycerides with low levels of high-density lipoprotein cholesterol were noted in three subjects (6). However, similar data have not been available from MAD patients with *ZMPSTE24* mutations. It is likely that, if the lipodystrophy progresses with puberty, our patients may also develop insulin resistance and dyslipidemia.

Deficiency of *Zmpste24* in mice causes accumulation of prelamin A and phenotypic features similar to those of MAD. Pendas et al. (31) reported partial lipodystrophy, muscular dystrophy, and cardiomyopathy in *Zmpste24*-deficient mice. Bergo et al. (32) reported that *Zmpste24*-deficient mice gained weight slowly and exhibited progressive hair loss, multiple spontaneous bone fractures, and muscle weakness. The toxicity is most likely due to accumulation of farnesylated prelamin A at the nuclear pore complexes, resulting in abnormal nuclear morphology and premature cell death (33). Therefore, Fong et al. (34) tested the efficacy of farnesyltransferase inhibitors (FTIs) in *Zmpste24*-deficient mice and reported improved body weight, grip strength, bone integrity, and percent survival at 20 weeks of age. These results suggest that FTIs decrease the accumulation of farnesylated prelamin A and may be potentially beneficial in patients with *ZMPSTE24* deficiency.

In summary, we report two Japanese siblings with MAD who harbor novel compound heterozygous mutations in the *ZMPSTE24* gene. In the 7-year-old girl, SC fat was almost totally absent with the exception of the cheeks, arms and thighs. Our patients had more severe phenotypes than those observed in MAD patients with *LMNA* mutations. Accumulation of farnesylated prelamin A may be responsible for toxicity in these patients.

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Fig. 1.

Photographs and radiographs of the patient 1 at 7 years of age. Frontal view of the total body (a) and lateral view of the head (b) and the left hand (e). Note the characteristic facies, lipodystrophy, skin atrophy with mottled hyperpigmentation, and fingertip rounding. Radiographs of the lateral view of the skull (c) and frontal view of the skull (d), left hand (f), and pelvis (g). Note the clinodactyly of the fifth finger, osteolysis of the distal phalanges, and delayed osseous maturation (f); hypoplastic mandible with dental overcrowding; delayed closure of cranial sutures and prominent wormian bone (d); and the osteolysis of the right femoral head (g).

Fig. 2.

Magnetic resonance imaging (MRI) of the various parts of body in patient 1 (**a–d**) and a 7-year-old normal healthy Japanese girl (**e–h**). (**a**) Coronal T1-weighted MRI around the hip joints showing deformity of right femoral head. SC fat around the pelvis and thigh region appears to be slightly reduced in patient 1 compared with the normal female (shown in **e**). SC fat in the abdomen appears to be normal in the patient. (**b**) Axial T1-weighted MRI at the level of heart showing reduction in SC fat in the thoracic region in patient 1 compared with the normal female (shown in **f**). Intrathoracic fat in the patient appears normal. (**c**) Axial T1-weighted MRI at the level of the thigh showing slightly reduced SC fat especially in the anterior and lateral regions in patient 1 compared with the normal female (shown in **g**). The fat in the intermuscular and bone marrow region appears normal. (**d**) Axial T1-weighted MRI at the level of pelvis showing reduction in SC fat in the anterior and posterolateral region in patient 1 compared with the normal female (shown in **h**). The osteolysis of the right femoral head is also evident in the patient.

Fig. 3. Mandibuloacral dysplasia (MAD) pedigree, sequence analysis and conserved motif of *ZMPSTE24*. (a) Pedigree of the MAD family with *ZMPSTE24* genotypes (MAD 3300.3). The mutations in *ZMPSTE24* are shown below the symbols. (b) Sequence electropherogram for mutations in exons 1 and 6 of *ZMPSTE24*. Identified mutations c.121C>T and c.743C>T are indicated by arrows, and wild-type sequences are shown above for comparison. (c) Alignment of partial *ZMPSTE24* sequences from human, dog, rat, mouse, zebra fish, *Caenorhabditis elegans*, Drosophila, Arabidopsis, and yeast. The residue Pro at position 248 is conserved among species. Also, the flanking residues F (Phe) and L (Leu) are also conserved except in *C. elegans* where F is conservatively changed to Y (Tyr). WT, wild type sequence.

Fig. 4. Yeast (*Saccharomyces cerevisiae*) halo assay for the functional analysis of the ZMPSTE24 mutants. The ZMPSTE24 mutation, Q41X, was inactive in complementing the yeast STE24 and produced no halo around the yeast colony; however, the mutation P248L in our assay appears as active as the wild-type (WT) ZMPSTE24.

Fig. 5.

Immunoblot analysis of cell lysates. (a) Expression of lamins A and C and prelamin A in lymphoblasts of the affected patients (MAD 3300.3 and 5). Shown is a representative immunoblot of cell lysates obtained from lymphoblasts of affected patients probed with amino-terminal-specific anti-lamin antibody, N-18, showing lamin C and mature lamin A in all lymphoblasts including an unrelated lymphoblast line as control and in HeLa cells. In affected patients (MAD 3300.3 and MAD 3300.5), an additional slower migrating immunoreactive species was detected, which corresponds to prelamin A. Same blot was stripped and reprobed with antibody to actin for equal protein loading. (b) Expression of lamins A and C and prelamin A in lymphoblasts of the parents (MAD 3300.1, father and MAD 3300.2, mother) and the affected patient (MAD 3300.3). There was no prelamin A band noted in the cell lysates from the parents. MAD, mandibuloacral dysplasia; MW, molecular weight.

Fig. 6. Immunofluorescence staining of lymphoblasts. Immunofluorescence images of lymphoblasts from affected patients and unrelated control. The cells were fixed and labeled as described in the Materials and methods and stained for lamin A (green fluorescence) and nuclear DNA (blue fluorescence). Shown are representative images for the lamin A/C staining in unaffected and affected patients. In affected lymphoblasts, the lamin A/C staining is less intense than that in the unaffected lymphoblasts. There is also significantly greater immunofluorescence in cells from patient 1 than in cells from patient 2. Magnification $\times 20$.