Table 3 Summary of possible pathogenic mutations

Gene	Nucleotide change	Amino acid change	NCBI ID	dbSNP135	Allele frequency in 1000GENOME	Allele frequency in ESP6500	Allele frequency in Japanese control	PolyPhen-2 prediction (score)	PROVEAN prediction (score)	Pathogenicity	Family	Reference
ACTG1	c.802G>A	p.G268S	NM_001199954.1	None	-	0	0/192	Probably damaging (0.998	Deleterious (-4.504)	Possible	1	
POU4F3	c.1007delC	p.A336Vfs	NM_002700.2	None	-	0	0/192	-	-	Possible	2	
SLC26A5	c.390A>C	p.R130S	NM_198999.2	None	-	0	0/192	Benign (0.443)	Deleterious (-4.813)	Possible	3	
SLC26A5	c.209G>A	p.W70X	NM_198999.2	None	-	0	n.t*.	-	-	Possible	3	
SIX1	c.328C>T	p.R110W	NM_005982.3	rs80356459	No info	0	n.t.	Probably damaging (1.000)	Deleterious (-7.775)	Causative	4	35
MYO7A	c.6478T>G	p.W2160G	NM_000260.3	None	-	0	0/192	Probably damaging (1.000)	Deleterious (-12.649)	Possible	5	
MYO7A	c.6439-2A>G (intron 51)	Splice mutation	NM_000260.3	None	-	0	0/192		-	Possible	5	
CDH23	c.719C>T	p.P240L	NM_022124.5	rs121908354	1/2183	0	n.t.	Probably damaging (1.000)	Deleterious (-3.051)	Causative	6	43
PCDH15	c.848G>A	p.R283H	NM_001142763.1	None	-	1/13005	0/192	Probably damaging (0.998)	Neutral (-1.918)	Possible	6	
USH2A	c.12431delC	p.A4144GfsX23	NM_206933.2	None	-	0	0/190		-	Possible	7	

*n.t. = not tested

Table 4 Summary of variants with uncertain pathogenicity

Gene	Nucleotide change	Amino acid change	NCBI ID	dbSNP135	Allele frequency in 1000GENOME	Allele frequency in ESP6500	Allele frequency in Japanese control	PolyPhen-2 prediction (score)	PROVEAN prediction (score)	Pathogenicity	Family	Reference
DFNA5	c.781C>T	p.R261X	NM_004403.2	None	-	0	0/192	-	-	Uncertain	2	
USH2A	c.1346G>A	p.R449H	NM_206933.2	None	-	0	5/378	Benign (0.017)	Neutral (-0.880)	Uncertain	7	

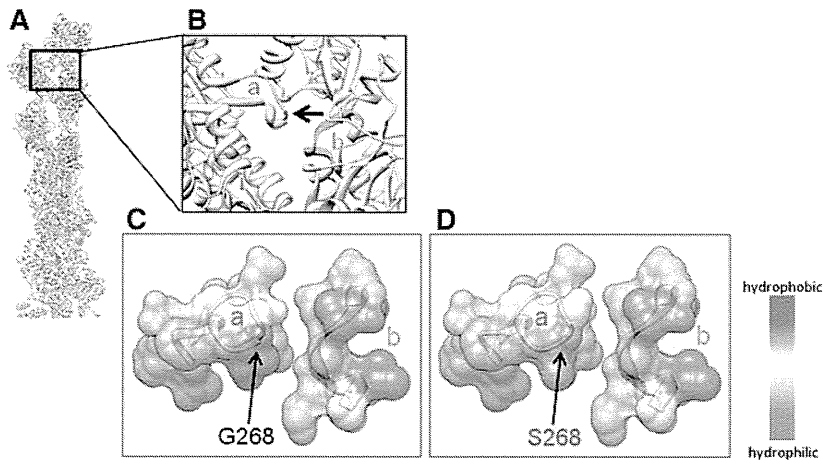

Figure 2 Molecular modeling of *ACTG* containing the p.G268S mutation. (A) Ribbon model of filamentous actin gamma 1. **(B)** Magnified ribbon model of filamentous actin gamma 1. Glycine residue 268 is shown in red and indicated by an arrow. Regions in yellow and green indicate the hydrophobic loop (262–274; a) and the corresponding interactive residues (281–289; b), respectively. **(C and D)** Vertical views of the regions a and b superimposed with predicted surface hydrophobicity in the wild type **(C)** and the p.G268S mutant **(D)**.

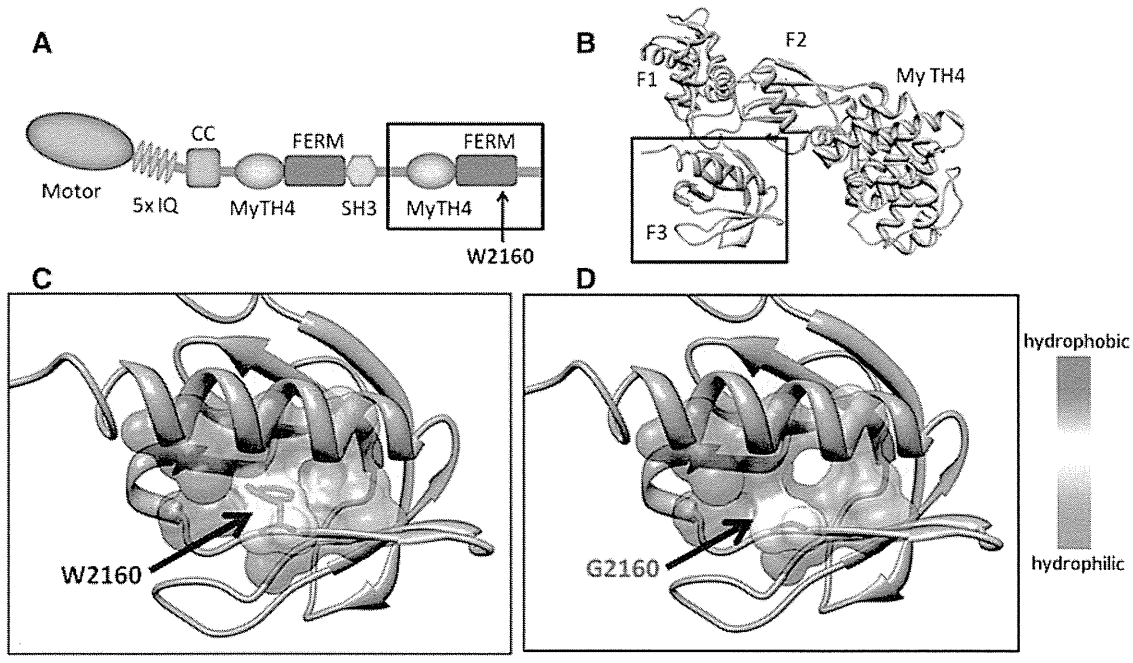

Figure 3 Molecular modeling of *MYO7A* containing the p.W2160G mutation. (A) Structural motif of myosin 7A. Tryptophan 2160 on the C-terminal 4.1 protein-ezrin-radixin-moesin (FERM) domain is indicated by an arrow. Motor, myosin motor domain; IQ, Isoleucine-glutamine calmodulin-binding motif; CC, coiled-coil domain; MyTH4, myosin tail homology 4 domain; SH3, Src homology 3 domain. **(B)** Ribbon model of the C-terminal FERM domain consisting of three subdomains (F1, F2, F3) and an MyTH4 domain. **(C, D)** Magnified ribbon model of the F3 subdomain superimposed with predicted surface hydrophobicity in the wild type **(C)** and the p.W2160G mutant **(D)**.

and *PCDH15* have been reported to be a digenic cause of hearing loss [46].

In family 7 (Figure 1G), subjects II:1 and II:2 with hearing loss did not have candidate mutations in the first 61 genes. Analysis of the additional 23 genes indicated a compound heterozygous *USH2A* variant or mutation, c.1346G >A(p.R449H) and c.12431delC (p.A4144GfsX23), in subjects with hearing loss, whereas subjects I:1 and II:2 with normal hearing had a heterozygous p.R449H variant and a heterozygous p.A4144GfsX23 mutation, respectively. *USH2A* is responsible for Usher syndrome 2A (OMIM 276901) [47]. Although *USH2A* with the p.R449H variant was not found on dbSNP135, 1000GENOME, or the Exome Variant Server, the allele frequency in Japanese control subjects with normal hearing was 1.3% (5/378).

In the remaining eight families, none of the detected variants co-segregated with hearing loss in the pedigrees (data not shown).

Discussion

In the present study we selected Japanese subjects that had hereditary hearing loss without *GJB2* mutations, mitochondrial mutations, enlarged vestibular aqueduct or auditory neuropathy-associated *OTOF* mutations, and we aimed to detect the spectrum of rare deafness genes in these patients. Targeted NGS for 84 deafness genes resulted in identification of candidate genes in 7 of 15 families and revealed the diverse spectrum of rare deafness genes in Japanese subjects with nonsyndromic hearing loss for the first time. This is the first report of mutations in *ACTG1*, *POU4F3*, and *SLC26A5* in Japanese families with hearing loss. Families 5, 6, and 7 appeared to have candidate mutations or variants in *MYO7A*, *CDH23*, *PCDH15*, and *USH2A*, all of which are associated with Usher syndrome [39,44,45,47]. Our results are in contrast to an NGS study of a different ethnic group [48], which showed *TMC1* mutations to be the prevalent candidate cause of hearing loss.

For the eight families without candidate genes, hearing loss could be attributable to mutations in non-captured regions including regulatory domains of the 84 genes, other unidentified deafness genes, unknown multigenic causes, copy number variations, or chromosomal structural change.

Double heterozygous mutations

In family 5, double heterozygous mutations of *CDH23* and *PCDH15* were detected as a candidate cause. This combination of double heterozygous mutations has been reported [46]. Cadherin 23 and protocadherin 15 consist of the upper and lower part of tip link, respectively, which is critical for proper function of mechanotransduction channels on the stereocilia of the sensory hair cells [49]. In addition, P240 of CDH23 is on the extracellular cadherin 1 domain, and R283 of PCDH15 is on the extracellular cadherin 2 domain, which are considered to interact with each other for tip-link bound [49], raising the possibility that the double heterozygous mutations could lead to a destabilized tip-link.

Additional findings of double heterozygous mutations associated with hereditary hearing loss have been reported for *KCNJ10* and *SLC26A4* [50] and for *FOXI1* and *SLC26A4* [51], and some mutated genes may have a modifying effect [52]. Although most NGS pipelines, including ours, focus on identifying monogenic causes of disease, development of a detection strategy for digenic and oligogenic causes of disease should be considered in the future.

Discrimination of mutations from variants

The key challenge for the diagnostic application of NGS is to distinguish causal alleles from the numerous nonpathogenic variants present in each individual. In the present study, for example, the high allele frequency of *USH2A* with the p.R449H variant in Japanese control subjects implied that pathogenicity of this variant was unlikely. Ethnic diversity of genetic variance has been reported in deafness genes such as *OTOF* [12] and *CDH23* [43,53], and integration of a database of genetic variants with allele frequencies in a specific ethnic group would increase the certainty of the causative nature of genetic mutations by filtering out variants that occur with high frequency. This would facilitate targeted NGS analysis for genetic diagnosis of hearing loss.

Additional files

Additional file 1: The 84 genes that were targeted for next-generation sequencing.

Additional file 2: Clinical features of family members.

Additional file 3: Audiograms of subjects with hearing loss in the seven families in which candidate genes were detected. Figure legend: Hearing level as a function of frequency in subject IV:2 from family 1 (A), subject III:3 from family 1 (B), subject IV:3 from family 2 (C), subject III:1 from family 2 (D), subject III:2 from family 2 (E), subject III:1 from family 3 (F), subject II:1 from family 4 (G), subject III:1 from family 5 (H), subject II:2 from family 6 (I), subject II:3 from family 6 (J), and subject II:2 from family 7 (K). Open circles with solid lines represent air conduction thresholds of the right ear; crosses with dotted lines represent air conduction thresholds of the left ear; [symbols represent bone conduction thresholds of the right ear;] symbols represent bone conduction thresholds of the left ear; arrows pointing to the bottom left represent scale-out hearing level of the right ear; arrows pointing to the bottom right represent scale-out hearing level of the left ear.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
HM and NS carried out capturing and sequencing the DNA samples, interpreted the data, and drafted the manuscript. CT carried out capturing and sequencing the DNA samples. AS and JK worked on DNA sequencing and interpreting the data. KN carried out molecular modeling of gene

products. KKosaki and TM designed the study and interpreted the data. NM, KKaga, and TM contributed to accumulation and interpretation of clinical data. TM finalized the manuscript. All authors read and approved the final manuscript.

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The novel *SLCO2A1* heterozygous missense mutation p.E427K and nonsense mutation p.R603* in a female patient with pachydermoperiostosis with an atypical phenotype

Running title: Mutations of *SLCO2A1* in Japanese pachydermoperiostosis

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Conflicts of interest

None declared.

Keywords

Pachydermoperiostosis, Primary hypertrophic osteoarthropathy, SLCO2A1, HPGD, Prostaglandin E2

DEAR EDITOR, Pachydermoperiostosis (PDP), or primary hypertrophic osteoarthropathy (PHO: MIM 167100), is a rare genetic disease affecting both skin and bones. The major diagnostic criteria include finger clubbing, periostosis, pachydermia, and cutis verticis gyrata (CVG). Additional symptoms, including sebaceous hyperplasia, hyperhidrosis, and arthropathy, have been reported.[1,2]

Uppal *et al.* discovered that a homozygous mutation in *HPGD*, which encodes 15-hydroxyprostaglandin dehydrogenase (15-PGDH), causes PHO and PDP.[3]

However, PHO and PDP are genetically heterogeneous. Exome analysis of PDP in Japanese, Chinese, Caucasian, and other races has revealed homozygous mutations in the solute carrier organic anion transporter family member 2A1 (*SLCO2A1*) gene, which encodes prostaglandin transporter (PGT).[4-8] Increased levels of prostaglandin E2 (PGE2) resulting from defective degradation contribute to the pathogenesis of PHO and PDP. A genetic defect in either *SLCO2A1* or *HPGD* can cause PHO and PDP.

In this study, we describe the first observation of a *SLCO2A1* mutation in a female patient.

A 67-year-old woman was referred for *SLCO2A1* mutation analysis. At the age of 43, she developed myelopathy of unknown aetiology. She received rehabilitation therapy without medication. A neurologist had examined her muscle weakness at the Th7 level on the right side following a diagnosis of suspected multiple sclerosis. At the age of 64, she had multiple seronegative arthralgias but no serious problems. She was referred to Tohoku Kouseinenkin Hospital because of recurring arthralgia and was treated with methotrexate and prednisone. She responded favourably to the medication with alleviation of the pain and decreased serum levels of C-reactive protein. Physical examination revealed finger clubbing and swelling of the large joints, as seen in panels (b) and (c) in Fig. 1. No skin manifestations, including facial

coarseness or greasiness, and no hyperhidrosis were observed. Marked thickening of the scalp (cutis verticis gyrata, CVG) was not evident. Radiological examination showed the presence of periostosis of the diaphysis of the tibia and fibula (Fig. 1d). No hydrarthrosis was evident. A diagnosis of possible incomplete type of PDP or PHO was made because of minimal pachydermia. She had no history of peptic ulcers and anaemia. Diagnostic imaging and laboratory data revealed no evidence of secondary PDP. She has a son and a daughter who were healthy.

This study was approved by the ethics committee of the National Centre for Child Health and Development and Keio University School of Medicine. The participants provided written informed consents. All exons of *HPGD* and *SLCO2A1* along with sequences adjacent to the exon-intron borders were amplified, sequenced, and screened for mutations.[4] Serum and urinary levels of PGE2 were measured with a commercial enzyme immunoassay kit (Cayman, Cayman Biochemical, Ann Arbor, MI, USA).[4]

We identified compound heterozygous novel mutations c.1279G>A/p.E427K and c.1807C>T/p.R603* in *SLCO2A1* (Fig. 2). We also detected a heterozygous mutation c.1279G>A in her daughter, but she has not developed any triad of PDP.

In her seventh decade, the patient's atypical history showed minimal impact of pachydermia. Serum PGE2 was not detected and her urinary PGE2 was within normal limits (372 pg/ml). One of the mutations, c.1279G>A (p.E427K), is included within the region of a previously reported deletion, c.1279_1290del12 (p.E427_P430del).[4] Another mutation, c.1807C>T/p.R603*, is detected close to the C-terminus of PGT, resulting in a shortened predicted protein. The lost function in truncated PGT is consistent with the presence of the p.R603* mutation in another patient, who had the complete type of PDP (manuscript in preparation).

This patient is the first woman with PDP who had an *SLCO2A1* gene mutation. It is unlikely that the mild phenotype of P1 was due to the missense mutation p.E427K. A recent report on PDP in a Chinese family described a homozygous p.A286Qfs*35 frameshift mutation in a male proband who had PDP.[7] Two of the proband's sisters were also homozygous for p.A286Qfs*35, but at ages 42 and 47, they had neither history nor findings suggestive of PDP.[7] Diggle *et al.*[6] reported that two women in two PDP families were homozygous for pathogenic *SLCO2A1* mutations. One had mild finger clubbing but no musculoskeletal or skin symptoms at 34 years of age. The other was asymptomatic at 19 years of age. Taken together, we propose that PDP resulting from SLCO2A1 defects is a sex-dependent autosomal recessive disease, and women homozygous for pathogenic *SLCO2A1* mutations may

develop late-onset PDP symptoms. The explanation of mild and low-frequency disease in women remains unclear. Hatano et al. suggested that reactivity to prostaglandin was milder in women than in men.[9] Ospina et al. reported that estrogen suppressed IL-1β-mediated induction of COX-2 pathway in rat cerebral blood vessels.[10] These data imply that decreased level of estrogen play a role in the sex-dependent pathogenesis. Further analyses will clarify this issue.

In conclusion, we have described the first female case of PDP with compound heterozygous *SLCO2A1* mutations. Her atypical history shows minimal pachydermia impact.

Acknowledgements

We thank the patient and her family members for their generous cooperation. We also thank Ms. A. Maruta for secretarial assistance and Ms. M. Furuhashi for her excellent technical assistance.

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Figure legends

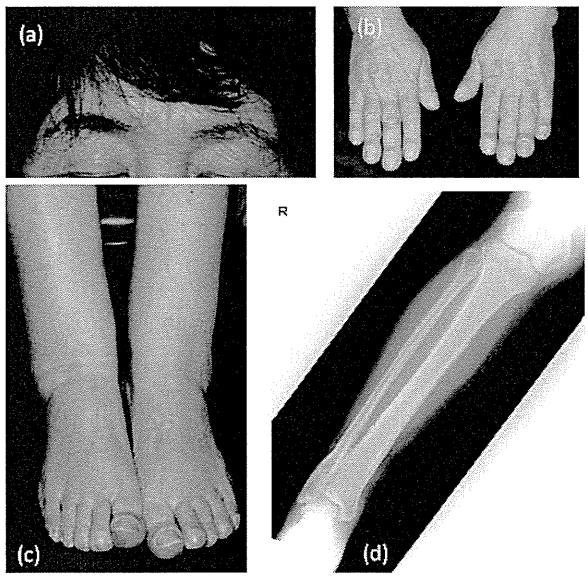
Fig. 1 Clinical features including a radiograph

Panel (a) shows the facial appearance of the patients. Furrowing of the forehead and greasiness of facial skin are negligible. Panel (b) shows digital clubbing. Panel (c) shows clubbing of toes and cylindrical enlargement of the legs. Panel (d) shows periosteal hyperostosis of the tibia and fibula.

Fig. 2 Two novel mutations in *SLCO2A1* were identified by the Sanger method

CodonCode Aligner (CodonCode Corporation, Centerville, MA, USA) was used to assemble sequences and detect mutations.

(a) One non-synonymous mutation: c.1279G>A/p.E427K (P1). (b) The premature stop codon mutations: c.1807C>T/p.R603*.



Niizeki et al:
Fig 1

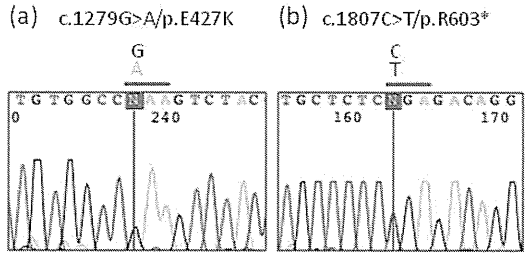


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Fig2

RESEARCH ARTICLE

AMERICAN JOURNAL OF medical genetics PART A

Multiple Café au Lait Spots in Familial Patients With *MAP2K2* Mutation

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Recent advances in genetic diagnostic technologies have made the classic disease nosology highly complicated. This situation is exemplified by rasopathies, among which neurofibromatosis type 1 and Noonan syndrome represent prototypic entities. The former condition is characterized by multiple café au lait spots and neurofibromas, while the latter is characterized by distinct facial features, webbed neck, congenital heart disease, and a short stature. On rare occasions, the features of both neurofibromatosis and Noonan syndrome co-exist within an individual; such patients are diagnosed as having neurofibromatosis–Noonan syndrome. Here, we report familial patients with multiple café au lait spots and Noonan syndrome-like facial features. A mutation analysis unexpectedly revealed a mutation in *MAP2K2* in both the propositus and his mother. The propositus fulfilled the diagnostic criteria for neurofibromatosis type 1, but his mother did not. Their phenotype was not consistent with that of cardio-facio-cutaneous syndrome, which is classically known to be associated with *MAP2K2* mutations. The mother of the propositus had cervical cancer at the age of 23 years, consistent with the oncogenic tendency associated with rasopathies. The phenotypic combination of multiple café au lait spots and Noonan syndrome-like facial features suggested a diagnosis of neurofibromatosis–Noonan syndrome. Whether this condition represents a discrete disease entity or a variable expression of neurofibromatosis type 1 has long been debated. The present observation suggests that some perturbation in the RAS/MAPK signaling cascade results in multiple café au lait spots, a key diagnostic phenotype of rasopathies, although the exact mechanism remains to be elucidated.

Key words: cafe au lait spots; rasopathies; MAP2K2; neurofibromatosis type 1; Noonan syndrome; neurofibromatosis–Noonan syndrome

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INTRODUCTION

A classic genetic syndrome is defined based on a combination of distinctive phenotypic features and causative genes. However, recent advances in molecular diagnostic technology have revealed significant phenotypic overlaps among classic syndromes, making genetic disease nosology more complicated than ever [Viskochil, 2011].

The RAS/mitogen activated protein kinase (MAPK) pathway is essential for the regulation of the cell cycle and differentiation. Somatic mutations in the RAS/MAPK signaling cascade can cause cancers, whereas germline mutations are responsible for several rare genetic conditions such as neurofibromatosis type 1 (OMIM 162200), Noonan (OMIM 163950), LEOPARD (OMIM 151100), Costello (OMIM 218040), and cardio-facio-cutaneous (CFC; OMIM 115150) syndromes. Given the considerable phenotypic and molecular overlaps among these conditions caused by germline RAS/MAPK mutations, they are collectively termed "rasopathies" [Tidyman and Rauen, 2009; Gripp and Lin, 2012].

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Abbreviations: CFC, cardio-facio-cutaneous; NFNS, neurofibromatosis–Noonan syndrome; MAPK, mitogen activated protein kinase.

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Among rasopathies, neurofibromatosis type 1 and Noonan syndrome represent prototypic entities: neurofibromatosis type 1 is an autosomal dominant disorder caused by loss-of-function mutations in the *NF1* gene on chromosome 17q11.2. This relatively common genetic condition is characterized by multiple café au lait spots and neurofibromas. Noonan syndrome is caused by heterozygous mutations in the *PTPN11*, *SOS1*, *KRAS*, *RAF1*, *BRAF*, *NRAS*, *CBL*, and *MAP2K1* genes and is characterized by hypertelorism, ptosis and low-set ears, webbed neck, congenital heart disease, chest deformities, postnatal reduced growth, and cryptorchidism [Cirstea et al., 2010; Martinelli et al., 2010; Tartaglia et al., 2010]. In rare cases, features of neurofibromatosis type 1 and Noonan syndrome co-exist, and such patients are classified as having neurofibromatosis–Noonan syndrome (NFNS) (OMIM 601321) [Allanson et al., 1985; Abuelo and Meryash, 1988]. Since patients with mutations in the *NF1* and *PTPN11* genes can present as having NFNS [Bertola et al., 2005; De Luca et al., 2005], it has long been debated whether NFNS is a discrete entity [Opitz and Weaver, 1985; Carey, 1998]. Here, we report familial patients with multiple café au lait spots and Noonan syndrome-like facial features who carried mutations in *MAP2K2* (*MEK2*), a component of the RAS/MAPK signaling cascade.

CLINICAL REPORT

The propositus was born at term via vaginal delivery in a breech position, with a birth weight of 3,535 g (+1.3 SD) and a length of 46.3 cm (−1.3 SD). He was noted as having multiple café au lait spots at birth. At the age of 6 years, he attended regular school, and a physical examination showed a short stature with a height of 107.5 cm (−2.0 SD) and a weight of 19.3 kg (−0.7 SD), multiple (>6) café au lait spots, axillary and inguinal frecklings, hypertelorism, downward-slanting palpebral fissures, and a webbed neck (Fig. 1A). No Lisch nodules were present. An echocardiogram was normal. The results of a brain magnetic resonance imaging examination at the age of 2 years were normal. The propositus' biological mother had multiple (>6) café au lait spots and no signs of intellectual disability. Her past medical history was significant for cervical cancer, for which she had undergone a total hysterectomy at the age of 27 years. At the age of 31 years, her height was 158 cm (−0.1 SD), and her weight was 54 kg (+0.3 SD). She had multiple café au lait spots, but did not exhibit any intertriginous frecklings (Fig. 1B).

MOLECULAR ANALYSIS

We performed target-selected resequencing using a custom-designed mutation analysis panel (SureSelect XT-Auto; Agilent Technologies, Santa Clara, CA) [manuscript in preparation], which included 100 common genes described in a classic textbook of dysmorphology: Smith's Recognizable Patterns of Human Malformation [Jones, 2006]. Included within the gene list were major molecules in the RAS/MAPK signaling cascade, including *NF1*, *PTPN11*, *SOS1*, *KRAS*, *RAF1*, *BRAF*, *NRAS*, *CBL*, *SHOC2*, *MAPK1*, *MAP2K1*, and *MAP2K2*. This panel was run on a next-generation sequencer (MiSeq; Illumina, Inc., San Diego, CA). After

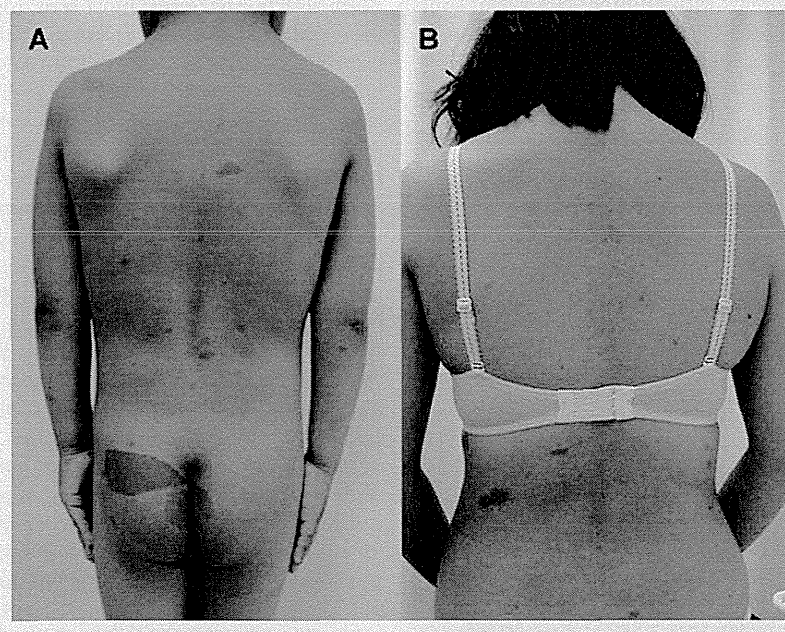

FIG. 1. Multiple café au lait spots on the propositus and his mother. Note the scattered multiple café (>6) au lait spots with well-demarcated margins in the propositus (A) and his mother (B) and the webbed neck in the propositus (A).

we aligned the sequencing reads to the reference human genome sequence (hs37d5) using BWA [Li and Durbin, 2009], local realignment around indels and base quality score recalibration were performed using Genome Analysis Toolkit software [McKenna et al., 2010]. Duplicate reads were removed using Picard (http://picard.sourceforge.net). This analysis revealed that the propositus and his mother were heterozygous for a missense mutation in exon 6 of *MAP2K2* (c. 667A>G, p.Met223Val). This p.Met223Val mutation was a novel variant that is not present in the dbSNP137, 1,000 genomes, ESP6500, or our in-house Japanese SNPs dataset. We confirmed the mutation detected in the propositus and his mother using Sanger sequencing and the following primers: forward, cctcacagcctgaaatggtt; reverse, agagcagcagggaggagag. In silico functional evaluations of the p.Met223Val substitution in MAP2K2 using five different prediction programs suggested that this mutation is pathogenic (PhyloP, "conserved" [score 0.9932]; PolyPhen2, "probably damaging" [0.8690]; SIFT, "damaging" [0.9900]; MutationTaster, "disease_causing" [1.0000]; and LRT, "deleterious" [1.0000]). p.Met223Val is evolutionarily conserved among many species and is located between two sites, p.Ser222 and p.Ser226, that are phosphorylated by RAF family proteins [Alessi et al., 1994] (Fig. 2). In addition, a crystal structure analysis indicated that the amino acid at position 223 is a substrate-binding residue [Ohren et al., 2004]. Therefore, p.Met223Val would alter the activity of MAP2K2, affecting downstream signaling. Direct sequencing of the *NF1* gene did not reveal any pathologic mutations in either the propositus or his mother.

DISCUSSION

Here, we report familial patients with multiple café au lait spots and Noonan syndrome-like facial features who carried mutations in *MAP2K2*. The identification of the *MAP2K2* mutations had diagnostic and therapeutic implications. The maternal history of cervical cancer at a very young age, that is, 27 years, suggested that this specific mutation, that is, p.Met223Val, may predispose an individual to cancer. This observation was consistent with the effects of somatic gain-of-function mutations in *MAP2K2* in melanoma patients [Nikolaev et al., 2012]. Although her cervical cancer was successfully resected, MEK inhibitors may be useful as a therapeutic option in the case of cancer recurrence.

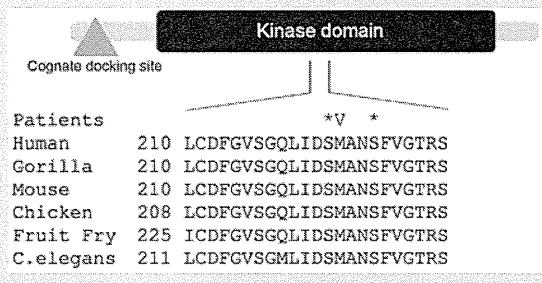

FIG. 2. Schematic representation of the MAP2K2 structure and partial amino acid sequence alignment from the propositus, human reference, and other species. Note that the amino acid sequence alignments in humans and other species were highly conserved. The p.Met223Val in the presently reported patients is marked at the top of the alignments as "V." Note that p. Met223Val is located between two sites, p.Ser222 and p.Ser226 (indicated as asterisks) located in the center of the kinase domain and that are phosphorylated by RAF proteins.

In retrospect, the diagnostic journey based on the patients' phenotype was rather challenging. The presence of multiple café au lait spots is a cardinal feature of neurofibromatosis type 1. Indeed, the propositus fulfilled the diagnostic criteria for neurofibromatosis type 1, but his mother did not. More specifically, the mother, who was 31 years of age, did not exhibit intertriginous freckling. The absence of this finding during the third decade of life practically excludes a diagnosis of neurofibromatosis type 1. Instead, the propositus had Noonan syndrome-like facial features, and his mother had cervical cancer at a very young age. This phenotypic constellation did not point to a specific disease entity, but strongly suggested that they had some form of rasopathies. The targeted mutation analysis panel consisting of the causative genes of major dysmorphic syndromes, including major molecules in the RAS/MAPK signaling cascade, successfully identified pathologic mutations in *MAP2K2*.

The identification of the causative gene in *MAP2K2* was rather unexpected. Typically, the germline mutations in *MAP2K2* have been associated with a CFC syndrome phenotype [Rodriguez-Viciana et al., 2006]. The classic phenotypic features of CFC syndrome include a relatively severe degree of developmental delay, heart defects, and eczematous skin, together with Noonan syndrome-like facial features [Narumi et al., 2007]. Interestingly, the presently reported patients did not have any of the above-mentioned features except for the Noonan-like facial features. Furthermore, the presence of multiple (>6), but not 2 or fewer, café au lait spots was suggestive of a cutaneous feature in neurofibromatosis type 1 [Siegel et al., 2011]. A review of familial patients with *MAP2K2* mutation confirmed a heterogeneous clinical picture consisting of learning disability, pulmonic stenosis, and café au lait spots (Table I) [Rauen et al., 2010; Linden and Price, 2011]. Overall, the phenotypic features of the family, including the Noonan syndrome-like facial features and multiple café au lait spots, in the absence of cardiac abnormalities or intellectual disability can be regarded as NFNS. The vertical transmission of the trait in the family is also compatible with NFNS [Quattrin et al., 1987].

The observation that *MAP2K2* mutation can lead to the NFNS phenotype casts another informative piece to the long-standing controversy of whether NFNS represents a discrete disease entity or a variable expression of neurofibromatosis type 1 [Opitz and Weaver, 1985]. We have shown that NFNS is heterogeneous at a clinical and a molecular level, as Carey exactly pointed out in his editorial 15 years ago [Carey, 1998]. What kind of perturbation in the RAS/MAPK signaling cascade results in café au lait spots, a key diagnostic phenotype in rasopathies, remains to be elucidated.