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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Flow chart of our variant detection and copy number analysis scheme.

Figure S2. Insufficient coverage of reads in two genes rich in repetitive sequences.

Mutations in *KLHL40* Are a Frequent Cause of Severe Autosomal-Recessive Nemaline Myopathy

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Nemaline myopathy (NEM) is a common congenital myopathy. At the very severe end of the NEM clinical spectrum are genetically unresolved cases of autosomal-recessive fetal akinesia sequence. We studied a multinational cohort of 143 severe-NEM-affected families lacking genetic diagnosis. We performed whole-exome sequencing of six families and targeted gene sequencing of additional families. We identified 19 mutations in *KLHL40* (kelch-like family member 40) in 28 apparently unrelated NEM kindreds of various ethnicities. Accounting for up to 28% of the tested individuals in the Japanese cohort, *KLHL40* mutations were found to be the most common cause of this severe form of NEM. Clinical features of affected individuals were severe and distinctive and included fetal akinesia or hypokinesia and contractures, fractures, respiratory failure, and swallowing difficulties at birth. Molecular modeling suggested that the missense substitutions would destabilize the protein. Protein studies showed that *KLHL40* is a striated-muscle-specific protein that is absent in *KLHL40*-associated NEM skeletal muscle. In zebrafish, *klhl40a* and *klhl40b* expression is largely confined to the myotome and skeletal muscle, and knockdown of these isoforms results in disruption of muscle structure and loss of movement. We identified *KLHL40* mutations as a frequent cause of severe autosomal-recessive NEM and showed that it plays a key role in muscle development and function. Screening of *KLHL40* should be a priority in individuals who are affected by autosomal-recessive NEM and who present with prenatal symptoms and/or contractures and in all Japanese individuals with severe NEM.

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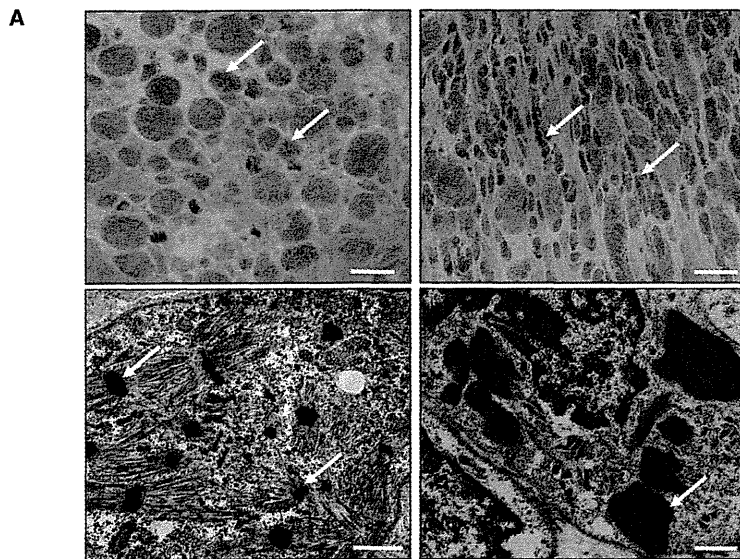
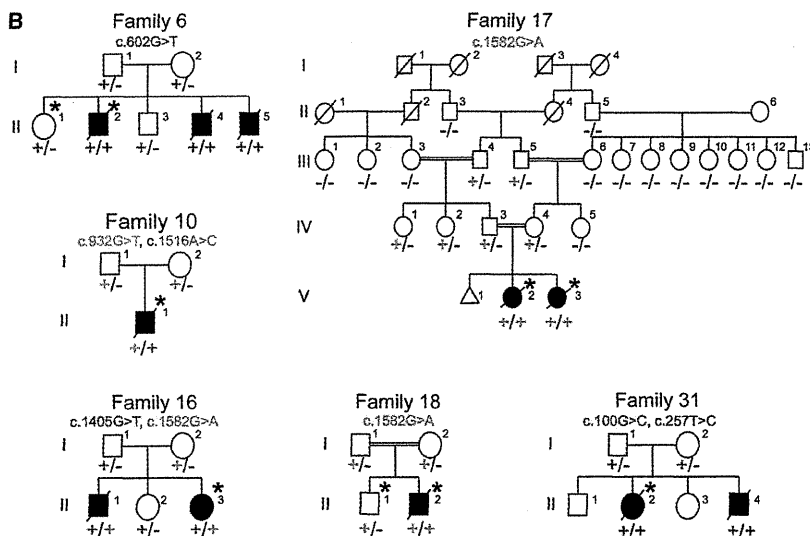


Figure 1. Family Pedigrees and Light and Electron Microscopy of Muscle Biopsies

(A) Modified Gomori trichrome (upper) and electron microscopy (lower) of muscle biopsies from affected individuals of families 15 (right) and 20 (left). Abnormal variation in fiber size, together with many small myofibers and sometimes increased connective tissue, and the presence of numerous red- or purple-stained nemaline bodies (arrows) can be seen (upper panels). Numerous nemaline bodies with varying sizes and shapes and a lack of normal myofibrils are visible by electron microscopy (arrows). Scale bars represent 20 μm for modified Gomori trichrome and 1 μm for electron microscopy.

(B) Pedigrees for the families in which exome sequencing and analysis were performed on the probands. Asterisks indicate the individuals whose DNA was analyzed by exome sequencing. Segregation of the mutations identified in each pedigree is shown.



pathway.¹⁰ Nevertheless, some forms of NEM remain genetically unsolved.

One such subtype, which has long been recognized,^{11,12} has apparent autosomal-recessive inheritance and is characterized by severe weakness, in utero presentation of fetal akinesia or hypokinesia and associated abnormalities, and muscle biopsy often showing numerous small nemaline bodies, sometimes only visible by electron microscopy and frequently with virtually no normal myofibrils remaining (“miliary NEM” Figure 1A and Figure S1, available online). We aimed to identify genetic causes of

these severe NEM cases by using a combination of linkage analysis, or homozygosity mapping, SNP array, and whole-exome sequencing (WES) in selected families. We have identified loss-of-function mutations in *KLHL40* as a frequent cause of severe NEM and have shown through functional studies that *KLHL40* is crucial for myogenesis and skeletal-muscle maintenance.

Subjects and Methods

Subject Details and Ethics

We recruited 143 genetically unresolved severe-NEM-affected families from large congenital-myopathy cohorts in major centers around the world (Boston, Helsinki, Perth, and Tokyo). All individuals within the cohorts were diagnosed with NEM on the basis of muscle-biopsy findings.

Written informed consent was obtained for participation in this study, which was approved by the Human Research Ethics

Introduction

Nemaline myopathy (NEM) is a common form of nondystrophic congenital myopathy and is defined clinically by skeletal-muscle dysfunction and pathologically by the presence of nemaline bodies within myofibers.^{1,2} Typical clinical symptoms include hypotonia, muscle weakness of proximal dominance, respiratory insufficiency, and feeding problems. Congenital onset is usual, but a wide variation in age of onset and disease severity is recognized. Mutations in seven genes are known to cause NEM (NEM1–NEM7).^{1,2} Six of these encode sarcomere-thin-filament proteins or associated proteins: *ACTA1* (MIM 102610),³ *CFL2* (MIM 601443),⁴ *NEB* (MIM 161650),⁵ *TNNT1* (MIM 191041),⁶ *TPM2* (MIM 190990),⁷ and *TPM3* (MIM 191030);⁸ the seventh, *KBTBD13* (kelch-repeat- and BTB-[POZ]-domain-containing 13 [MIM 613727])⁹ is involved in the ubiquitin proteasome

Committee of the University of Western Australia (UWA), the ethics committee of the Children's Hospital of the University of Helsinki, Yokohama City University School of Medicine, and the Boston Children's Hospital institutional review board. The UWA Animal Ethics Committee approved animal studies.

Microscopy

Light microscopy and electron microscopy of biopsies was performed as previously described.¹³

Whole-Genome SNP Genotyping, Linkage Analysis, and WES

Genotyping was performed for families 6 and 18 with the use of the HumanOmniExpress BeadChip Kit (Illumina) and Infinium II Assay Workflow (Illumina) at the Institute for Molecular Medicine Finland (FIMM). Data were analyzed with PLINK v.1.07. Multiple large homozygous regions were identified, but none included known myopathy-associated genes. WES was performed on one healthy and one affected sibling from family 6 and the proband from family 18 with the SeqCap EZ Human Exome Library v.2.0 exome system (Nimblegen, Roche Diagnostics). Coverage depths were 31- to 62-fold. Variant quantification was performed with the FIMM Variant Calling Pipeline v.1.0 and the Integrative Genomics Viewer (IGV, Broad Institute of MIT and Harvard). All known and heterozygous SNPs were excluded. Healthy siblings' genotypes were used for the exclusion of shared homozygous variants.

Five individuals from family 16 were genotyped with the Human Mapping 10K XbaI 142 2.0 array (Affymetrix) and GeneChip Genotyping Analysis Software (Gtypev4.1). Parametric linkage analysis was performed with Allegro v.2 with a fully penetrant autosomal-recessive model. WES was performed on the proband with the use of the SureSelect Human All Exon 50 Mb Kit (Agilent Technologies) and sequenced in one lane on a GAIIX platform (Illumina) with 108 bp paired-end reads. Reads were aligned to the UCSC Genome Browser (GRCh37/hg19) with Novoalign (Novocraft Technologies). Mean coverage depth was 59-fold. Single-nucleotide variants and small indels were identified with GATK UnifiedGenotyper and filtered according to the Broad Institute's Best Practices guidelines v.3. Variants registered in dbSNP132 were filtered. The filter-passed variants were annotated with ANNOVAR. Only genes with homozygous variants or more than two variants located in the candidate linkage regions were included.

Family 17 was genotyped with the HumanCytoSNP-12 BeadChip (Illumina). MERLIN was used for performing linkage analysis on a subset of 14,514 SNPs.¹⁴ WES was performed for the proband from family 10 and for both siblings from family 17 as described.¹⁵ Coverage depth was 61- to 97-fold. Variants were called with LifeScope 2.5 (Life Technologies) and filtered with ANNOVAR¹⁶ against ENCODE GENCODE v.11 (October 2011 freeze, GRCh37).¹⁷ Two custom variant-filtering steps were used: (1) one against the 1000 Genomes database (February 2012 release) (variants with a minor allele frequency > 0.5% were excluded) and (2) one against the dbSNP135 common database.

Family 31 (BOS74) was one in a cohort of 59 NEM-affected families who underwent WES by the Intellectual and Developmental Disabilities Research Center Core Next-Gen Sequencing Facility of Boston Children's Hospital and Harvard Medical School in collaboration with Axeq Technologies, Complete Genomics, Integrated Genetics (LabCorp), and the Boston Children's Hospital Gene Partnership. Exome sequencing was performed with the Illu-

mina HiSeq 2000 platform. Reads were mapped with the Burrows-Wheeler Aligner (v.0.5.8). SNPs and indels were called with SAMtools (v.0.1.7). Data analysis and variant calling were performed with the Broad GATK Best Practices for identification of SNPs and small indels. Annotated variants were filtered against dbSNP135, the 1000 Genomes Project database (October 2011 edition), and the National Heart, Lung, and Blood Institute (NHLBI) Exome Sequencing Project Exome Variant Server (EVS).

Sequencing

Bidirectional Sanger sequencing of *KLHL40* (RefSeq accession number NM_152393.2) was performed on biobanked DNA from additional probands with severe NEM and their family members in Boston, Helsinki, Perth, Yokohama, and Tokyo. Identified variants were then screened in all available family members. Primer sequences and conditions are available upon request. For detection of the c.1582G>A (p.Glu528Lys) mutation in normal Japanese controls, high-resolution melting (HRM) analysis with and without the spike-in method¹⁸ was performed on LightCycler 480 System II (Roche Diagnostics). If samples showed any aberrant melting patterns, Sanger sequencing was performed for confirmation of the mutation.

LOD Scores

Where possible, MERLIN was used for calculating LOD scores for individual families.¹⁴

Expression Analysis on Human cDNAs

TaqMan quantitative real-time PCR analyses were performed with cDNAs of human adult (Human MTCPanel I, #636742, Clontech Laboratories) and fetal (Human Fetal MTC Panel, #636747, Clontech Laboratories) tissues.¹⁹ Predesigned TaqMan probe sets for human *KLHL40* (*KBTD5*, Hs00328078_m1, Applied Biosystems) and human β -actin (*ACTB*, 4326315E, Applied Biosystems) were used. PCR was performed on a Rotor-Gene Q (QIAGEN) (conditions are available upon request) and analyzed with the Rotor-Gene Q Series Software by the $2^{-\Delta\Delta C_t}$ method. Relative concentrations of cDNA were normalized to concentrations obtained from the hearts.

Calculations of the Free-Energy Change upon Amino Acid Substitutions

Molecular structures were drawn with PyMOL. FoldX v.3.0 beta²⁰ was used through a graphics interface as a plugin for the YASARA molecular viewer.²¹ Crystal structures of the kelch domain of human *KLHL40* (Protein Data Bank [PDB] code 4ASC) and the BTB (bric-a-brac, tram-track, broad-complex)-BACK (BTB and C-terminal kelch) domain of human *KHLH11* (PDB code 3I3N) were energy-minimized with the RepairPDB command implemented in FoldX and subsequently with the BuildModel command for mutagenesis. Protein stabilities were calculated by the Stability command, and the free-energy changes were estimated by subtraction of the free-energy value of the wild-type protein from those of the altered proteins. The procedure was repeated three times for each substitution, and the resultant data were presented as an average value with SDs.

Immunoblotting and Immunohistochemistry

SDS-PAGE and immunoblotting were performed as described.^{22,23} For protein studies, C2C12 myoblasts and myotubes were grown and prepared for immunoblotting and immunofluorescence as

described.²³ For KLHL40 immunoblots, the Human Protein Atlas (HPA) rabbit polyclonal KLHL40 (KBTBD5) antibody from Sigma was used (HPA024463 [1:2,500 dilution]). Immunostaining of human and mouse muscle samples was performed as described^{1,3,23} with a KLHL40 antibody (KBTBD5; HPA024463 [1:100 dilution]).

Zebrafish Studies

In Situ Hybridization

Digoxigenin probes for *klhl40a* and *klhl40b* were generated by cDNA amplification of 1,340 and 694 bp sequences, respectively (Table S1). *In situ* hybridizations were performed as described previously.²⁴

Morpholino Microinjection

Antisense translation-blocking morpholinos (Table S1) for *klhl40a* (*klhl40a*-MO) and *klhl40b* (*klhl40b*-MO and *klhl40b*-MO2) were coinjected into 1- to 2-cell-stage embryos at a final concentration of 0.25 or 0.5 mM. Morpholino efficacies were tested by immunoblotting for Khl40.

Zebrafish Immunohistochemistry

Immunohistochemistry of zebrafish embryos was performed as described^{24,25} with myosin heavy chain (MHC) antibody (F59 [1:20 dilution] or A4.1025 [1:10 dilution]; Developmental Studies Hybridoma Bank) and α -actinin (1:100 dilution; Sigma) and filamin C (1:100 dilution; Sigma) antibodies, and Alexa-Fluor-488-conjugated phalloidin (1:100 dilution; Molecular Probes) was used for labeling F-actin. Immunoreactivity was detected with an Alexa-Fluor-594-conjugated anti-mouse secondary antibody diluted in blocking buffer (1:200).

Statistical Analyses

Statistical analyses of clinical features were carried out with SPSS Statistics 19 (IBM) software. Individuals for whom information for a clinical feature was not available were excluded from the analysis of that feature. Either Chi-square tests or Fisher's exact tests were applied for comparing each phenotypic variable between different genotypes. $p < 0.05$ was considered statistically significant.

Results

WES identified homozygous or compound-heterozygous mutations in *KLHL40* (kelch-like family member 40; also known as *KBTBD5* [kelch-repeat- and BTB-(POZ)-domain-containing 5] and *SYRP* [sarcosynapsin]) in six NEM-affected families (families 6, 10, 16–18, and 31; Figure 1B and Table 1). Subsequent screening of *KLHL40* by Sanger sequencing in additional probands with severe NEM resulted in the identification of a total of 19 variants (4 frameshifts, 12 missense mutations, 2 nonsense mutations, and 1 splice site) in 28 (19.6%) apparently unrelated families (Table 1) from the cohort of 143 families affected by severe NEM. In addition, 129 probands with milder NEM were screened, but no *KLHL40* mutations were identified in this cohort, confirming that *KLHL40* mutations are most likely exclusive to cases of severe NEM.

In all cases where it was possible to test unaffected parents, siblings, and extended family, the mutations cosegregated with disease in an autosomal-recessive fashion (Figure 1B), giving a combined LOD score of 5.66 (Table

1). All mutations were either absent from the NHLBI EVS and the 1000 Genomes database²⁶ or present at low frequencies in the heterozygous state (Table 1). In five additional NEM-affected families, only single *KLHL40* variants were identified (Table S2); the significance of these variants in these individuals remains unclear.

In Japanese persons, *KLHL40* mutations are the most common cause of this severe form of NEM (13/47 [~28%]) as a result of a founder effect with the c.1582G>A mutation. Given that this mutation was present in Turkish, Kurdish, and Japanese families, we completed a haplotype analysis of Japanese and Turkish families (families 16 and 17) but did not identify a common haplotype between them (Figure S2). HRM with confirmatory Sanger sequencing of 510 normal Japanese individuals revealed a heterozygous c.1582G>A mutation in one individual. Therefore, the mutant-allele frequency in the Japanese population was estimated to be 0.0098. According to the equation described by Kimura and Ota²⁷ and under the assumption of 25 years per generation, the age of this mutation is calculated to be 4,900 years old.

The identified *KLHL40* mutations were scattered throughout all exons (Table 1 and Figure 2A) encoding mostly conserved residues (Figure S3). To investigate disease mechanisms, all substitutions except p.Arg311Leu were mapped to the crystal structures of the kelch domain of human *KLHL40* and the BTB-BACK domain of human kelch-like protein 11 (*KLHL11*; Figures 2B and 2C and Figure S4). p.Arg311Leu (c.932G>T) was predicted to be in the structurally flexible region, a linker of nonconserved amino acids connecting the BACK and kelch domains (Figure S7D), and was therefore excluded from structural consideration. All the modeled substituted residues are involved in intramolecular interactions, and thus the substitutions would most likely destabilize the hydrophobic cores of the BTB-BACK domain (p.Leu86Pro [c.257T>C], p.Val194Glu [c.581T>A], and p.Trp201Leu [c.602G>A]), the kelch domain (p.Pro397Leu [c.1190C>T], p.His455Arg [c.1364A>G], and p.Gly469Cys [c.1405G>T]), the β sheet (p.Thr506Pro [c.1516A>C] and p.Ala538Pro [c.1612G>C]), or the hydrogen bonds between the main chain and side chain (p.Asp34His [c.100G>C] and p.Glu528Lys [c.1582G>A]) or between side chains (p.Glu588Lys [c.1762G>A]) (Figures S5–S7). The p.Pro397Leu and p.Glu588Lys substitutions appear to be conservative for the hydrophobic core and hydrogen bonding, respectively. The former substitution is predicted to affect the polyproline II helix conformation (residues 396–399; Figure S6A). The calculated free-energy change for most substitutions was estimated to be over 2.0 kcal/mol (Figure 2D), which is typically associated with destabilization of domain folds.²⁸ These analyses suggested that most *KLHL40* missense mutations impair protein stability.

To investigate *KLHL40* expression and *KLHL40* abundance, we performed quantitative RT-PCR and immunoblotting of human and mouse tissues. *KLHL40* transcripts

Table 1. *KLHL40* Mutations by Family, Individual LOD Scores, Ethnicity, and Population-wide Incidence

Family	Exon(s)	Mutation		LOD Score	Ethnicity	Incidence from EVS (1 st ; 2 nd)	Incidence from 1000 Genomes (1 st ; 2 nd)
		Nucleotide Change	Amino Acid Change				
Family 31 ^a	1	c.[100G>C];[257T>C]	p.[Asp34His];[Leu86Pro]	0.6	Vietnamese	ND; ND	ND; ND
Family 2	1	c.[134delC];[134delC]	p.[Pro45Argfs*19]; [Pro45Argfs*19]	NA	Italian	NA	ND
Family 3	1	c.[270C>G];[270C>G]	p.[Tyr90*];[Tyr90*]	NA	Turkish	ND	ND
Family 5	1	c.[581T>A];[581T>A]	p.[Val194Glu];[Val194Glu]	0.6	Israeli	ND	ND
Family 6 ^a	1	c.[602G>T];[602G>T]	p.[Trp201Leu];[Trp201Leu]	1.454	Turkish	ND	ND
Family 7	1	c.[602G>A];[602G>A]	p.[Trp201*];[Trp201*]	NA	Norwegian	ND	ND
Family 9	1	c.[790delC];[790delC]	p.[Arg264Alafs*59]; [Arg264Alafs*59]	0.25	Turkish	NA	ND
Family 10 ^a	1 and 4	c.[932G>T];[1516A>C]	p.[Arg311Leu];[Thr506Pro]	NA	Chinese	ND; ND	ND; ND
Family 34	2 and 6	c.[1190C>T];[1762G>A]	p.[Pro397Leu];[Glu588Lys]	NA	Turkish	ND; ND	ND; A = 2 and G = 2,184
Family 12	2 and 4	c.[1270_1272delinsAGATC AAGGT];[1582G>A]	p.[Asp424Argfs*23]; [Glu528Lys]	NA	Japanese	NA; ND	ND; ND
Family 13	2 and 4	c.[1281_1294delCTGCCTGG ACTCGG];[1582G>A]	p.[Cys428Hisfs*12]; [Glu528Lys]	NA	Korean	NA; ND	ND; ND
Family 14	3	c.[1364A>G];[1364A>G]	p.[His455Arg];[His455Arg]	NA	Turkish	ND	ND
Family 15	3	c.[1405G>T];[1405G>T]	p.[Gly469Cys];[Gly469Cys]	NA	Japanese	ND	ND
Family 16 ^a	3 and 4	c.[1405G>T];[1582G>A]	p.[Gly469Cys];[Glu528Lys]	0.727	Japanese	ND; ND	ND; ND
Family 17 ^a	4	c.[1582G>A];[1582G>A]	p.[Glu528Lys];[Glu528Lys]	1.654	Turkish	ND	ND
Family 18 ^a	4	c.[1582G>A];[1582G>A]	p.[Glu528Lys];[Glu528Lys]	0.125	Kurdish	ND	ND
Family 19	4	c.[1582G>A];[1582G>A]	p.[Glu528Lys];[Glu528Lys]	0.25	Kurdish	ND	ND
Family 20	4	c.[1582G>A];[1582G>A]	p.[Glu528Lys];[Glu528Lys]	NA	Japanese	ND	ND
Family 21	4	c.[1582G>A];[1582G>A]	p.[Glu528Lys];[Glu528Lys]	NA	Japanese	ND	ND
Family 22	4	c.[1582G>A];[1582G>A]	p.[Glu528Lys];[Glu528Lys]	NA	Japanese	ND	ND
Family 23	4	c.[1582G>A];[1582G>A]	p.[Glu528Lys];[Glu528Lys]	NA	Japanese	ND	ND
Family 24	4	c.[1582G>A];[1582G>A]	p.[Glu528Lys];[Glu528Lys]	NA	Japanese	ND	ND
Family 25	4	c.[1582G>A];[1582G>A]	p.[Glu528Lys];[Glu528Lys]	NA	Japanese	ND	ND
Family 26	4	c.[1582G>A];[1582G>A]	p.[Glu528Lys];[Glu528Lys]	NA	Japanese	ND	ND
Family 27	4	c.[1582G>A];[1582G>A]	p.[Glu528Lys];[Glu528Lys]	NA	Japanese	ND	ND
Family 28	4	c.[1582G>A];[1582G>A]	p.[Glu528Lys];[Glu528Lys]	NA	Japanese	ND	ND
Family 29	4/5	c.[1608-1G>A];[1608-1G>A]	NA	NA	Turkish	ND	ND
Family 30	5	c.[1612G>C];[1612G>C]	p.[Ala538Pro];[Ala538Pro]	NA	Turkish	ND	ND

The individual pedigree LOD scores are given where possible. This table also shows the incidence of the mutations reported within the NHLBI EVS and the 1000 Genomes browser. Abbreviations are as follows: NA, not available; and ND, not detected.

^aFamilies for whom WES was performed.

and their encoded proteins were exclusive to developing and adult skeletal muscle (Figures 3A–3C) and more abundant in fetal muscle than in postnatal muscle (Figure 3C). Confocal microscopy suggested that KLHL40 might localize to the sarcomeric A-band (Figure 3D and Figure S8), a region not previously linked to NEM. Immunoblotting showed that KLHL40 is absent or of low abundance in *KLHL40*-associated NEM muscle (Figure 3E), even for persons harboring two missense mutations (F10 and

F17). Immunohistochemistry confirmed that KLHL40 was absent or very scarce in *KLHL40*-associated NEM myofibers (Figure 3F).

We further investigated Klhl40 function in zebrafish. The zebrafish genome contains two orthologs of *KLHL40*: *klhl40a* and *klhl40b*, which have 57% (*klhl40a*) and 55.7% (*klhl40b*) amino acid similarity to human KLHL40. RT-PCR demonstrated expression of both *klhl40* genes at 24 and 48 hr postfertilization (hpf) (Figure S9A). In adult

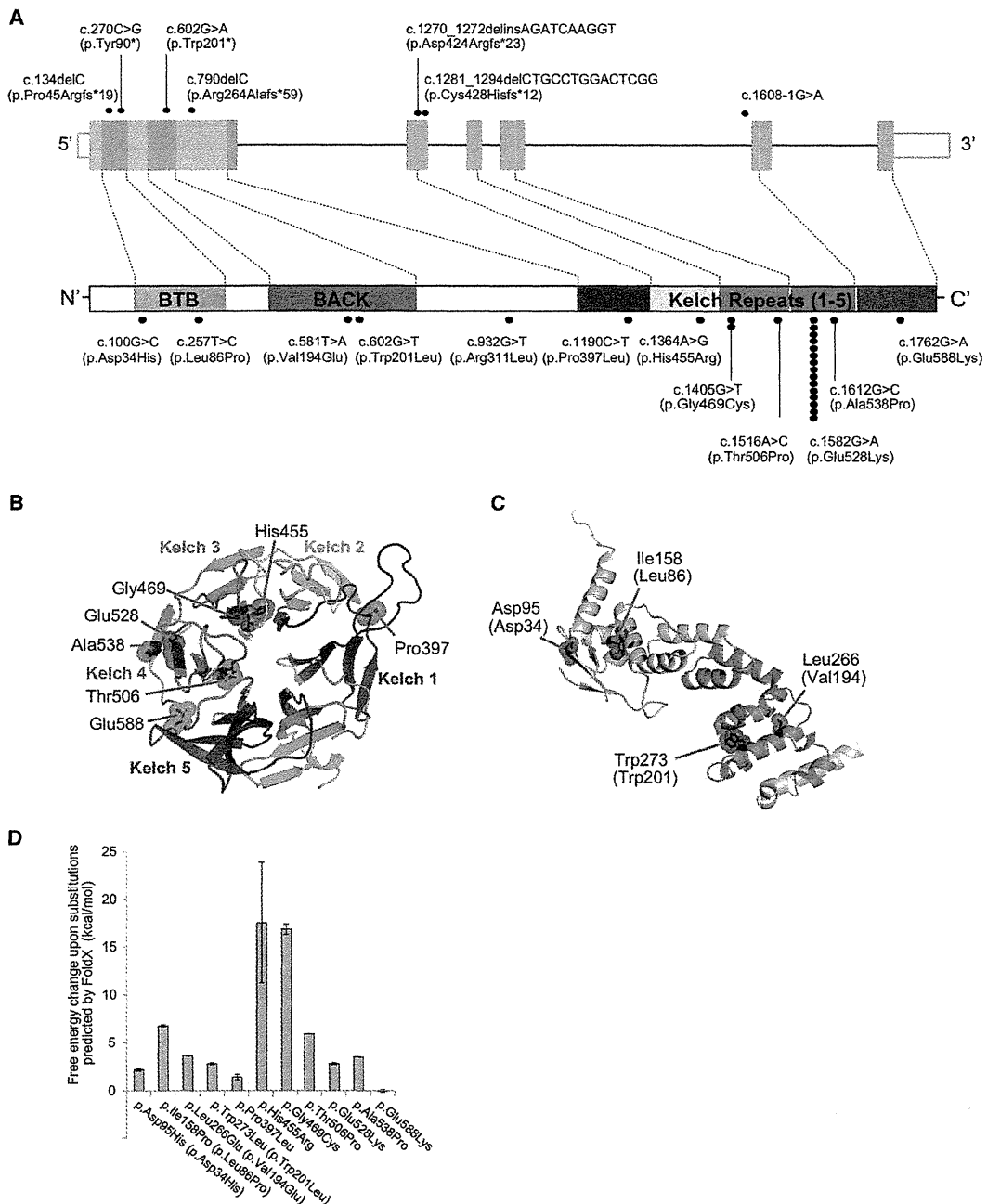


Figure 2. Mutations Identified in Our Cohort and the Structural Modeling of the Missense KLHL40 Substitutions
 (A) Schematic presentation of the genomic structure of *KLHL40* (upper) and its encoded protein, KLHL40, with the BTB-BACK domain and kelch repeats (lower). The localization of mutations and substitutions identified is depicted with dots, and the number of dots for each mutation or substitution indicates the number of times it was found. Most substitutions occurred at conserved amino acids. The dots above *KLHL40* indicate truncating mutations, and those below *KLHL40* indicate missense mutations.
 (B and C) Structural modeling of the missense KLHL40 substitutions. The crystal structures of the (B) kelch domain of KLHL40 and the (C) BTB-BACK domain of KLHL11 and the location of the substitutions are shown. p.Pro397Leu, p.His455Arg, p.Glu469Cys, p.Thr506Pro, p.Glu528Lys, p.Ala538Pro, and p.Glu588Lys map to the kelch repeats (B), p.Asp34His and p.Leu86Pro map to the BTB domain, and p.Val194Lys and p.Trp201Leu map to the BACK domain (C). The side chains of the mutated residues are shown as sticks with space-filling spheres in red. α helices, β sheets, and loops are drawn as ribbons, arrows, and threads, respectively. Each kelch repeat (B) is color coded in the kelch domain, and the BTB and BACK domains (C) are colored pink and green, respectively. Molecular structures were drawn with PyMOL.
 (D) The calculated free-energy changes resulting from the missense substitutions in the kelch domain of human KLHL40 and the BTB-BACK domain of human KLHL11 were predicted by FoldX. Data are presented as the mean \pm SD. Residue numbers used in (C) and (D) refer to human KLHL11, and those corresponding to human KLHL40 are in parentheses.

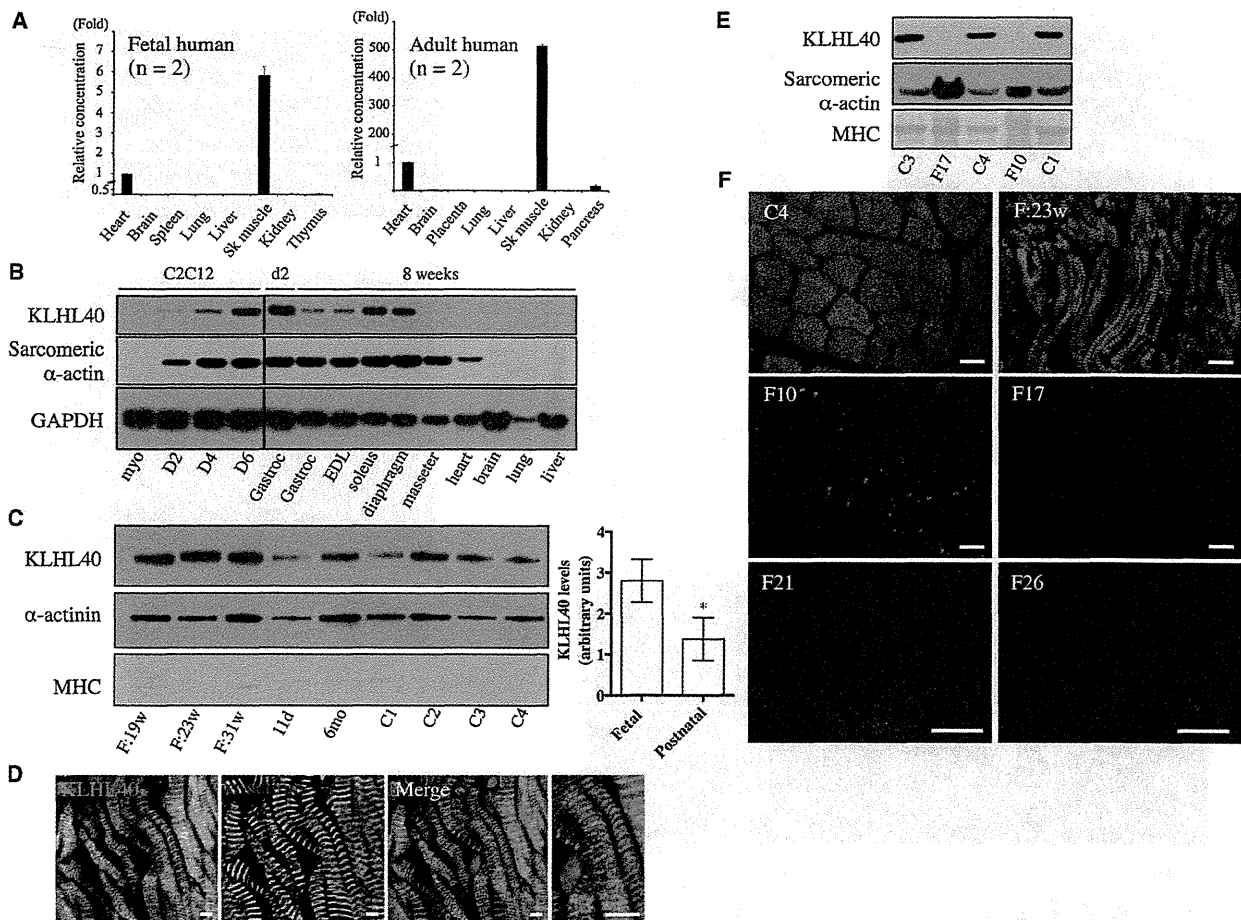


Figure 3. KLHL40 Expression in Human and Mouse Tissues

(A) Taqman quantitative real-time PCR analysis of cDNA from adult or fetal human tissues. Error bars represent the SD. The following abbreviation is used: Sk, skeletal.

(B) KLHL40 levels in C2C12 cells and mouse tissues (HPA, top panel) and immunoblotting for sarcomeric α -actin (clone 5C5, middle panel) and GAPDH (lower panel). Lanes are as follows: myo, C212 myoblasts; D2, myotubes on day 2 of differentiation; D4, myotubes on day 4 of differentiation; D6, myotubes on day 6 of differentiation; Gastroc (left), C57BL/6 postnatal day 2 (d2) gastrocnemius; Gastroc (right), C57BL/6 8-week-old gastrocnemius; and EDL (extensor digitorum longus) to liver, C57BL/6 8-week-old tissues. For all mouse tissue lysates, samples were pooled from three different mice.

(C) On the left is KLHL40 expression in human skeletal muscle (HPA, top panel), immunoblotting for α -actinin (clone EA-53, middle panel), and Coomassie staining of MHC band (bottom panel). Lanes are as follows: F:19w, 19-week-old fetus; F:23w, 23-week-old fetus; F:31w, 31-week-old fetus; 11d, 11-day-old neonate; 6mo, 6-month-old baby; and C1–C4, healthy adult controls of 19–42 years of age. On the right, KLHL40 intensity normalized to MHC for fetal muscle is 3.34 ± 0.92 ($n = 3$) versus 1.37 ± 0.21 ($n = 6$) for postnatal skeletal muscle. * $p = 0.023$, unpaired two-tailed t test. Error bars represent the SEM.

(D) Single Z-plane confocal microscopy showing localization of KLHL40 (green) and α -actinin (red) in a longitudinal section of skeletal muscle from a 31-week-old fetus; costaining with Hoechst (blue) is also shown (Merge). Scale bars represent 5 μ m.

(E) Immunoblotting shows that KLHL40 is absent in *KLHL40*-associated NEM muscle (II-1 from family 10 [F10] and V-2 from family 17 [F17]) compared with healthy control muscle (C1, C3, and C4). Coomassie staining of the MHC band (bottom panel) and immunoblotting for sarcomeric α -actin (clone 5C5, middle panel) indicate similar or greater loading for the *KLHL40*-associated NEM samples compared with control samples.

(F) Immunofluorescence for KLHL40 in a human 23-week-old fetal skeletal muscle sample (F:23w), an adult healthy control (C4), and *KLHL40*-associated NEM muscle biopsies (II-1 from family 10 [F10], V-2 from family 17 [F17], family 21 [F21], and family 26 [F26]). Scale bars represent 50 μ m.

zebrafish, *klhl40a* was most abundant in the skeletal muscle and heart and *klhl40b* was most abundant in the skeletal muscle (Figure S9A). At the 16 and 24 hpf stages, expression of both genes was restricted to the muscle precursor cells in the somites (Figure 4A). We knocked down zebrafish *klhl40a* and *klhl40b* with antisense morpholino

oligonucleotides (*klhl40a*-MO, *klhl40b*-MO, and *klhl40b*-MO2) (Figures S9B and S10A). Embryos injected with *klhl40a*-MO, *klhl40b*-MO, and *klhl40a*-MO/40b-MO (double morpholinos) showed a curved trunk and small head at 48 hpf (Figures 4B and 4C). A normal phenotype resulted from 5 bp mismatched morpholinos (5mis-MOs).

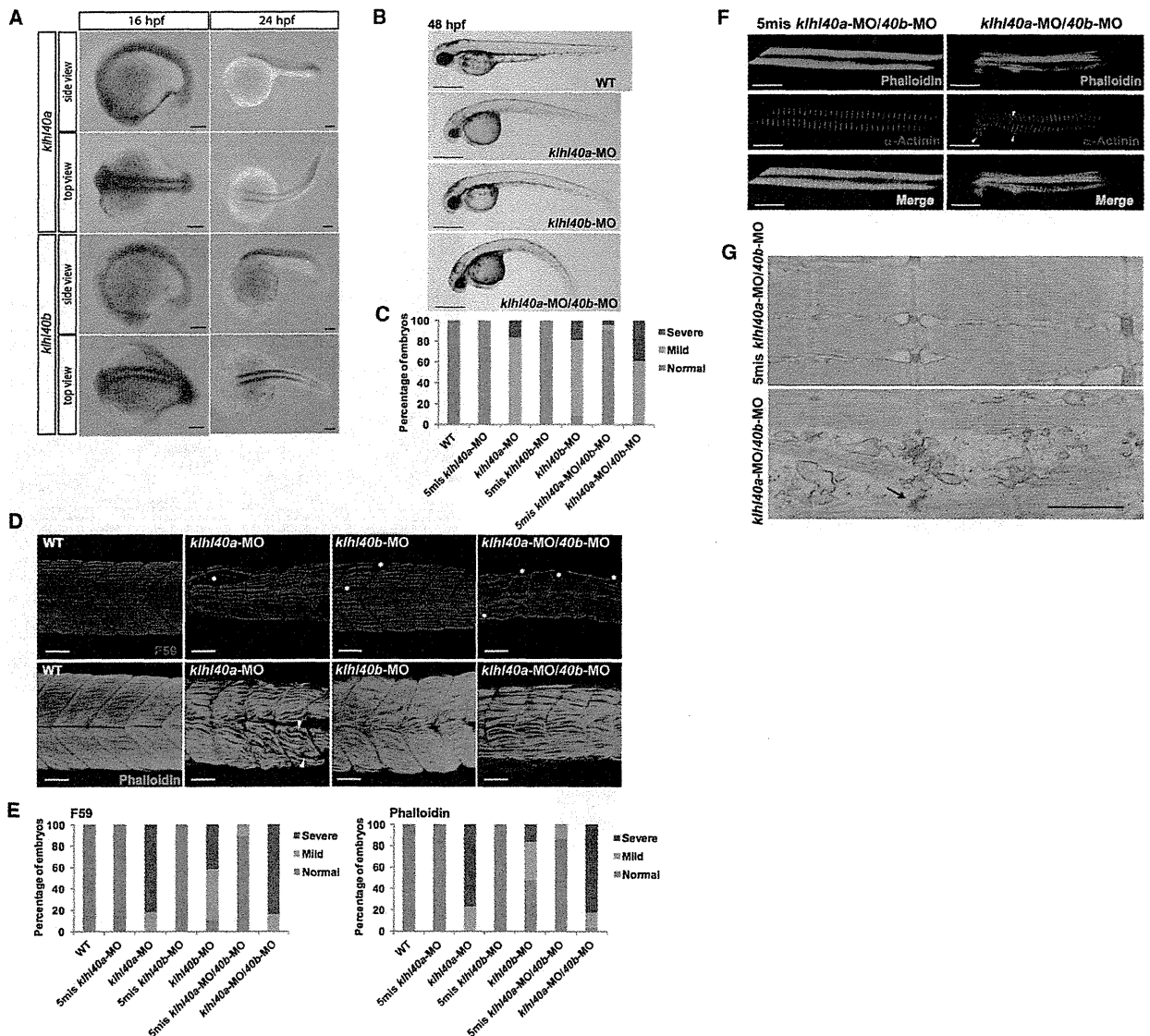


Figure 4. Expression and Function of *klh40* in Zebrafish

(A) In situ hybridization demonstrates that expression of both *klh40a* and *klh40b* is restricted to the skeletal muscle at 16 and 24 hpf. (B) Gross morphology of uninjected embryos (WT) and embryos injected with *klh40a*-MO, *klh40b*-MO, and *klh40a*-MO/*40b*-MO. Lateral views of MO-injected embryos (4 ng) at 48 hpf are shown. Scale bars represent 500 μ m.

(C) Percentage of embryos categorized in phenotypic classes after injection with the 5mis-MO control, *klh40a*-MO, *klh40b*-MO, or *klh40a*-MO/*40b*-MO. We categorized the phenotypes at 48 hpf into normal (normal appearance), mild (curved trunk), and severe (tail defect and severe development delay) ($n = 111$ – 130).

(D) Knockdown of *klh40a*, *klh40b*, or both resulted in severe disruption of the skeletal muscle: fibers appeared wavy, and there were extensive gaps between fibers in contrast to the densely packed and aligned fibers of the controls. Maximum-intensity projection images from a confocal image series followed immunolabeling with a myosin antibody (F59, upper panels) at 36 hpf and F-actin (lower panels) at 72 hpf.

(E) Embryos injected with 5mis-MO, *klh40a*-MO, *klh40b*-MO, or *klh40a*-MO/*40b*-MO were categorized phenotypically on the basis of the presence of myofiber detachment affecting one to two somites (mild) or multiple (three or more) somites (severe) ($n = 25$ – 44).

(F) Double-labeled immunofluorescence was performed on isolated myofibers from 72 hpf embryos with the use of phalloidin (green) and α -actinin (red). Frequent areas of aberrant α -actinin accumulation were detected in *klh40a*-MO/*40b*-MO myofibers (arrowheads).

(G) Electron microscopy of 72 hpf myofibers. A 5mis-MO-injected embryo shows correctly aligned sarcomeres and T-tubules (upper panel). A *klh40a*-MO/*40b*-MO-injected embryo (lower panel) shows disarranged myofibrils with widened Z-disks (arrow), but thin filament lengths are unchanged. The scale bar represents 0.7 μ m.

We analyzed slow myofibers in more detail by immunostaining slow myosin heavy chains (Figure 4D, upper panels). *klh40* morphants showed disruption of muscle

patterning with an irregular, wavy appearance of the striated myofibers and extensive gaps between the myofibers (Figures 4D and 4E and Figure S10B) and a greatly

Table 2. Summary of Clinical Features of NEM Individuals with *KLHL40* Mutations

	Individuals with <i>KLHL40</i> Mutations (n = 32 Cases from 28 Families)	
	Total	Percentage
Family history	17/28	60.7%
Consanguinity	10/28	35.7%
Prenatal Period		
Prenatal symptoms	24/29	82.8%
Fetal akinesia or hypokinesia	16/21	76.2%
Polyhydramnios	14/29	48.3%
Neonatal Period		
Respiratory function		
respiratory failure	28/29	96.6%
requiring ventilation	11/29	37.9%
Facial involvement		
weakness	23/23	100%
ophthalmoparesis	4/23	17.4%
mild dysmorphology	15/15	100%
Dysphagia		
with tube feeding or gastrostomy	13/24	54.2%
Muscle weakness		
with no spontaneous antigravity movements	13/29	44.8%
Contracture(s)	24/27	88.9%
Pathological fracture(s)	10/19	52.6%
Average age at death	5 months (n = 14)	
Average gestation age at birth	37 weeks (n = 27)	
Average birth weight	2,558 g (n = 26)	

Total numbers were calculated as the number of individuals with the clinical features over the total number of individuals whose medical records were available for each category.

diminished birefringence (Figure S10C). Isolated myofibers from *klhl40a*-MO/*40b*-MO fish, coimmunostained with phalloidin and an α -actinin antibody (Z-disk), showed disorganized and irregular patterns with small aggregates of α -actinin, suggesting nemaline bodies (Figure 4F). Aggregation of Z-disk material was also confirmed by immunostaining for filamin C in *klhl40a*-MO/*40b*-MO fish (Figure S11). Electron-microscopic analysis revealed disarranged myofibrils with widened Z-disks (Figure 4G). Fish injected with *klhl40a*-MO, *klhl40b*-MO, *klhl40b*-MO2, or *klhl40a*-MO/*40b*-MO2 (double morpholinos) exhibited sporadic muscle tremors, and coordinated swimming behavior was not observed (Movies S1 and S2). These results suggest that *Klhl40a* and *Klhl40b* are required for muscle development and function and that loss of either isoform in the early embryo is sufficient to impair normal mobility.

Detailed clinical records were collected and analyzed for 32 affected individuals from the 28 unrelated kindreds afflicted with *KLHL40* mutations. These individuals were from various ethnicities, such as European, Middle and Near Eastern, or Asian. Clinical features of individuals with *KLHL40* mutations were severe and distinctive (Table 2 and Table S3). Eighty-three percent of affected individuals showed prenatal symptoms, and 76% displayed fetal akinesia or hypokinesia. Most persons had severe respiratory compromise (97%), and approximately a third required ventilatory support (38%). Almost all affected individuals (96%) also had swallowing problems, and half required tube feeding or gastrostomy. Muscle weakness was severe. Forty-five percent of individuals had no spontaneous antigravity movement. Seventeen percent of affected individuals were also noted to have ophthalmoparesis, a relatively rare symptom in NEM. Multiple joint contractures and pathological bone fracture were other common features. Dysmorphic facial features and deformities of the chest, spine, fingers, and feet were also frequent. The average age of death was 5 months. Many families, including a previously described family (family 30 herein, cases 2–6 in Lammens et al.),¹¹ were consanguineous.

We further evaluated whether there are any genotype-phenotype correlations in *KLHL40*-associated NEM. We compared the clinical features of individuals according to the type of mutation they had (either two truncating mutations, one truncating mutation and one missense mutation, or two missense mutations) and the pattern of mutations (homozygous or compound heterozygous). No significant differences in frequencies of these clinical features were observed (data not shown). We also compared the clinical features of persons with the recurrent c.1582G>A genotype (either with this mutation [genotype G/A or A/A as group A] or without [genotype G/G as group G]). Prenatal symptoms, including fetal akinesia or hypokinesia, were frequently observed (73.3% in group A versus 92.9% in group G). Respiratory failure was common in both groups (100% in group A versus 92.9% in group G), but there were significantly fewer individuals requiring ventilation in group A than in group G (20.0% in group A versus 57.1% in group G; $p = 0.047$). Dysphagia was also common in both groups (100% in group A versus 90.0% in group G), but there were fewer persons requiring tube feeding or gastrostomy in group A than in group G, although the difference was not significant (42.9% in group A versus 70.0% in group G; $p = 0.127$). Facial weakness was observed in all affected individuals in both groups, but fewer individuals in group A had ophthalmoparesis (7.7% in group A versus 30.0% in group G; $p = 0.281$). All persons also had muscle weakness, but significantly fewer individuals in group A had the most severe form of muscle weakness with no antigravity movements (20.0% in group A versus 71.4% in group G; $p = 0.018$). Significantly fewer affected individuals in group A were deceased at the time of study than in group G (23.5% in group A versus 71.4% in group G; $p = 0.012$;

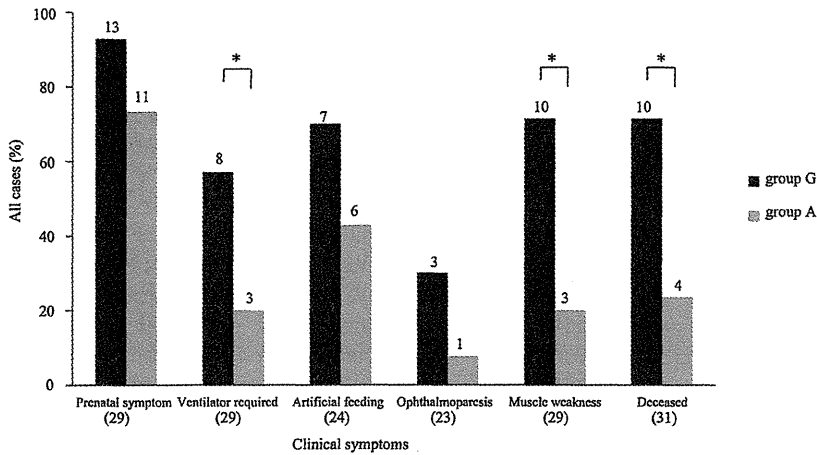


Figure 5. Correlation between the c.1582G>A (p.Glu528Lys) Mutation and Clinical Features

The clinical characteristics of NEM are shown for the two groups of affected individuals (32 total), either with the c.1582G>A (p.Glu528Lys) mutation (as group A) or without it (as group G). The numbers of total affected individuals with clinical records regarding either the presence or the absence of each characteristic are indicated below the bars, and the numbers of affected individuals in each group are indicated above the respective bars. Labels on the x axis are as follows: prenatal symptoms, individuals demonstrating either fetal akinesia or hypokinesia, polyhydramnios, or fetal edema or effusion; ventilator required, individuals with respiratory failure requiring ventila-

tion; artificial feeding, dysphagia-affected persons requiring tube feeding or gastrostomy; ophthalmoparesis, individuals with ophthalmoparesis along with facial weakness; muscle weakness, individuals with the most severe form of muscle weakness and demonstrating no antigravitatory movement; and deceased, individuals who were deceased at the time of study. Asterisks indicate that statistical significance was observed.

odds ratio = 8.125; 95% confidence interval = 1.62–40.75) (Figure 5). We further compared the clinical features of individuals of different ethnicities (either European or Asian descent) according to the c.1582G>A genotype, and similar tendencies were demonstrated (data not shown). There was, however, great variation in severity for individuals with or without the c.1582G>A genotype.

Discussion

We have described the identification of recessive *KLHL40* mutations in individuals with severe NEM from 28 unrelated families of various ethnicities. The c.1582G>A mutation was the most frequently detected mutation and was found in Japanese, Kurdish, and Turkish persons. However, comparison of haplotypes between a Japanese family and a Turkish family suggested that the mutation arose independently in these ethnic groups. We have shown several lines of evidence of the pathogenicity of the *KLHL40* mutations. The missense mutations occurred mostly in conserved functional domains within *KLHL40*, and they were predicted to destabilize the intramolecular interactions and thus impair protein stability. This was corroborated by the absence of *KLHL40* even in the skeletal muscle of individuals harboring two missense mutations. We have established a locus-specific database for *KLHL40* mutations at the Leiden Muscular Dystrophy Pages.

Expression of *KLHL40* in fetal and adult skeletal muscle indicates that *KLHL40* plays a role in both myogenesis and mature muscle. *KLHL40* appears to be more abundant in fetal skeletal muscle than in postnatal skeletal muscle and most likely accounts for the prevalence of in utero presentations in this NEM cohort. Perhaps *KLHL40* is more important for myogenesis than for muscle maintenance; this could account for the fact that the disease ranges so

much in severity, from some individuals' dying within hours of being born to others' surviving into adolescence. Our zebrafish studies have demonstrated that *Klhl40a* and *Klhl40b* are not required for the specification of muscle cells but rather for muscle patterning and function and that loss of either isoform in the early embryo is sufficient to impair normal mobility, supporting the involvement of *KLHL40* in NEM-associated fetal akinesia. It has previously been suggested that *KLHL40* is also important for muscle maintenance through the process of degeneration and regeneration.^{29,30} *Klhl40* is upregulated in myogenic precursors after cardiotoxin injury of mouse skeletal muscle, supporting a role for *Klhl40* in the response to muscle damage.²⁹ Studies of cattle muscle have shown increased *Klhl40* expression in another catabolic process, undernutrition, further suggesting a role for *KLHL40* in the stress response.³⁰

KLHL40 belongs to the superfamily of kelch-repeat-containing proteins that form characteristic β -propeller structures,³¹ which bind substrate proteins and are involved in a wide variety of functions. In humans, 71 kelch-repeat-containing proteins have been identified.³¹ The majority contain an N-terminal BTB domain (also known as the POZ [poxvirus and zinc finger] domain) and a BACK motif. Proteins containing both a BTB domain and a kelch repeat have previously been implicated in neuromuscular disease. A dominant *KLHL9* mutation causes an early-onset distal myopathy (distal myopathy 1 [MIM 160500]),³² and dominant *KBTBD13* mutations cause nemaline myopathy with cores (MIM 609273).⁹ We now show that *KLHL40*, encoding *KLHL40*, which contains both a BTB domain and a kelch repeat, is associated with autosomal-recessive neuromuscular disease. BTB domains function as substrate-specific adaptors for cullin 3 (Cul3),^{33,34} a component of the E3-ubiquitin-ligase complex. Both *KLHL9* and *KBTBD13* bind Cul3.^{10,32} MuRF1,

an E3-ubiquitin ligase, is known to be recruited to M-line titin and is thought to modulate myofibrillar turnover and the trophic state of muscle.³⁵ *KLHL40* appears to be present at the A-band and might be similarly involved through the ubiquitin-proteasome pathway.

We have characterized the severe and distinctive features of this disease as fetal akinesia or hypokinesia during the prenatal period, respiratory failure and swallowing difficulty at birth, contractures and fractures along with dysmorphic features, and in most cases, early death. We have also shown that persons with the recurrent c.1582G>A mutation tend to have relatively milder symptoms compared to those of individuals without c.1582G>A. However, the severity of the disease in persons with or without the c.1582G>A genotype varied greatly (for example, from death at 20 days to still being alive at 11 years for persons homozygous for the c.1582G>A genotype), suggesting modifying factors.

Fetal akinesias are clinically and genetically heterogeneous, and the majority of cases still remain genetically unsolved.³⁶ Primary muscle diseases account for up to 50% of such syndromes.³⁷ On the basis of our study, *KLHL40* mutations cause a significant proportion of severe NEM cases of fetal akinesia sequence and the disease shows worldwide prevalence. *KLHL40* should be considered when a clinician encounters an individual presenting with prenatal symptoms, such as fetal akinesia or hypokinesia, or clinical features and/or pathology of severe NEM at birth (especially miliary NEM, which was present in at least 20% of our *KLHL40*-mutation cases), along with an autosomal-recessive pattern of family history. Fractures are a relatively frequent presentation within this cohort, unlike other NEM cohorts, and should also be used for directing genetic screening of *KLHL40*. We show that *KLHL40* immunohistochemistry, immunoblotting, or genetic screening will identify the disease and thus allow genetic counseling for the affected individual's family.

In conclusion, this study associates loss-of-function *KLHL40* mutations with severe, often in utero, NEM. Many probands who do not harbor *KLHL40* mutations present with NEM in utero, suggesting further genetic heterogeneity. Clarification of *KLHL40* function and interactions might lead to a greater understanding of the pathogenesis of disease, the identification of other candidates for this severe form of NEM, and the investigation of possible therapies.

Supplemental Data

Supplemental Data include 11 figures, three tables, and two movies and can be found with this article online at <http://www.cell.com/AJHG>.

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Web Resources

The URLs for data presented herein are as follows:

1000 Genomes Project, <http://www.1000genomes.org/>

dbSNP, <http://www.ncbi.nlm.nih.gov/projects/SNP/>

Leiden Open Variation Database, www.LOVD.nl/KLHL40

NHLBI Exome Sequencing Project (ESP) Exome Variant Server, <http://evs.gs.washington.edu/EVS/>

Online Mendelian Inheritance in Man (OMIM), <http://www.omim.org>

PyMOL, <http://www.pymol.org>

RefSeq, <http://www.ncbi.nlm.nih.gov/RefSeq>

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MLL2 and KDM6A Mutations in Patients With Kabuki Syndrome

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Kabuki syndrome is a congenital anomaly syndrome characterized by developmental delay, intellectual disability, specific facial features including long palpebral fissures and ectropion of the lateral third of the lower eyelids, prominent digit pads, and skeletal and visceral abnormalities. Mutations in *MLL2* and *KDM6A* cause Kabuki syndrome. We screened 81 individuals with Kabuki syndrome for mutations in these genes by conventional methods ($n = 58$) and/or targeted resequencing ($n = 45$) or whole exome sequencing ($n = 5$). We identified a mutation in *MLL2* or *KDM6A* in 50 (61.7%) and 5 (6.2%) cases, respectively. Thirty-five *MLL2* mutations and two *KDM6A* mutations were novel. Non-protein truncating-type *MLL2* mutations were mainly located around functional domains, while truncating-type mutations were scattered through the entire coding region. The facial features of patients in the *MLL2* truncating-type mutation group were typical based on those of the 10 originally reported patients with Kabuki syndrome; those of the other groups were less typical. High arched eyebrows, short fifth finger, and hypotonia in infancy were more frequent in the *MLL2* mutation group than in the *KDM6A* mutation group. Short stature and postnatal growth retardation were observed in all individuals with *KDM6A* mutations, but in only half of the group with *MLL2* mutations. © 2013 Wiley Periodicals, Inc.

Key words: Kabuki syndrome; *MLL2*; *KDM6A*; mutation; genotype–phenotype correlation

INTRODUCTION

Kabuki syndrome (KS; OMIM 147920) is a multiple congenital anomaly syndrome that was originally reported by Niikawa et al. [1981] and Kuroki et al. [1981] (also known as Kabuki make-up syndrome or Niikawa–Kuroki syndrome). KS is diagnosed clinically by characteristic facial features, including long palpebral fissures and ectropion of the lateral third of the lower eyelids, postnatal growth impairment (short stature), developmental delay, intellectual disability, dermatoglyphic abnormalities, visceral and skeletal abnormalities, and immunological dysfunction. The prevalence of the disorder is estimated to be 1 in 32,000 live births [Niikawa et al., 1988]. Two genes have shown to be mutated in patients with KS: *MLL2* (myeloid/lymphoid or mixed-lineage leukemia 2; NM_003482.3) at 12q13.12 and *KDM6A* (lysine (K)-specific demethylase 6A; NM_021140.2) at Xp11.3 [Ng et al., 2010; Lederer et al., 2012; Miyake et al., 2013]. *MLL2* encodes a histone H3 lysine 4 (H3K4)-specific methyl transferase and *KDM6A* is a specific demethylase of histone H3 lysine 27 (H3K27) [Prasad et al., 1997; Lee et al., 2007]. They are both trithorax group proteins and bind each other [Schuettengruber et al., 2007]. These proteins are important for the chromatin state and transcription activation: *MLL2* methylates H3K4 and *KDM6A* removes the H3K27 trimethylation repressive mark

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[Dubuc et al., 2013]. The loss of *MLL2* or *KDM6A* function may lead to repressed transcription [Dubuc et al., 2013].

To our knowledge, there has been no comprehensive screen for mutations in these two genes in the same patient series. In this report, we performed a mutation screen of both genes in 81 patients with KS. We then evaluated the clinical features based on the genetic information.

MATERIALS AND METHODS

Samples

Eighty-one individuals clinically suspected to have KS were incorporated in this study: 77 Japanese, two Caucasians, one Belgian, and one Thai. They were all sporadic except for KMS-79, who had an affected sibling. Peripheral blood samples or saliva samples from the

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patients and their parents (when available) were collected with informed consent and DNA was extracted using a QuickGene-610L (Fujifilm, Tokyo, Japan) or Oragene-DNA kit (DNA Genotek, Inc., Ottawa, Canada) according to the manufacturer's instructions. This study included four previously reported patients (KMS-50, KMS-51, KMS-61, and KMS-71) [Tekin et al., 2006; Torii et al., 2009; Ito et al., 2012]. In addition, three patients with a *KDM6A* mutation were previously described as Patients 1, 2, and 3 by Miyake et al. [2013], and are named KMS-31, KMS-37, and KMS-65, respectively, in this report. This study was approved by the Institutional Review Board of Yokohama City University School of Medicine.

Mutation Screening

Fifty-eight patients (KMS-01 to KMS-69) were screened for *MLL2* mutations by the high-resolution melting (HRM) method using a LightCycler 480 System II (Roche Diagnostics, Indianapolis, IN) and subsequent Sanger sequencing. If an HRM curve pattern was different from those of controls, the DNA sample was Sanger sequenced on an ABI 3500xl or 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA) and the sequences were analyzed using Sequencher software version 4.10.1 (Gene Codes Corporation, Ann Arbor, MI). *KDM6A* was analyzed in samples with no *MLL2* mutation using HRM analysis and Sanger sequencing as above ($n = 37$). For male samples, genotyping using spike-in control male genomic DNA (10%) was performed to detect a hemizygous mutation. The latter 23 patients (KMS-70 to KMS-92), as well as 22 patients with no mutation in either gene detected by conventional methods, were analyzed by targeted resequencing as described in the following section. We judged a variant as pathogenic when it was previously reported to cause KS, or novel variant when it was not observed in unaffected parents or in in-house exome data ($n = 977$), dbSNP135, or EVS6500 (Exome Variant Server, NHLBI GO Exome Sequencing Project, Seattle, WA; <http://evs.gs.washington.edu/EVS/>; accessed March 1, 2013). In addition, the missense mutation predicted to be polymorphism by both of two predictions (Polyphen-2: <http://genetics.bwh.harvard.edu/pph2/> [Adzhubei et al., 2010] and MutationTaster: <http://www.mutationtaster.org/> [Schwarz et al., 2010]) was considered to be non-pathogenic. Parentage analysis was conducted for the patients only when the parental samples were available. TaKaRa Ex Taq and TaKaRa LA Taq (both Takara, Tokyo, Japan) were used for amplification. The primer sequences and PCR conditions are available on request. All pathological variants were confirmed by Sanger sequencing. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence (RefSeq NM_003482.3 for *MLL2*, RefSeq NM_021140.2 for *KDM6A*).

Targeted Resequencing of *MLL2* and *KDM6A* by Next-Generation Sequencing

Ion AmpliSeq Custom Panels (Life Technologies, Inc., Grand Island, NY) covering the entire coding region of *MLL2* and *KDM6A* were created via the Ion AmpliSeq Designer v1.2 (<https://ampliseq.com/browse.action>). Libraries were prepared using the Ion AmpliSeq Library Kit 2.0 (Life Technologies, Inc.), with 10 ng of genomic DNA for each primer pool (two pools for this

analysis). An Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, CA) and the associated High Sensitivity D1K Screen Tape (Agilent Technologies) were used to check the size distribution and the concentration of the DNA libraries. Emulsion PCR and enrichment steps were carried out using the Ion OneTouch 200 Template Kit v2 (Life Technologies, Inc.). The amplicon libraries were sequenced on an Ion Torrent Personal Genome Machine system using 314 or 316 chips, and bar-coding was applied with an Ion Xpress Barcode Adapters 1–16 Kit (all Life Technologies, Inc.). Torrent Suite 2.2 (Life Technologies, Inc.) was used for mapping, base calling, and variant calling. Sequences were annotated using SeattleSeq Annotation 134 (<http://snp.gs.washington.edu/SeattleSeqAnnotation134/>). All variants were confirmed by Sanger sequencing.

Whole Exome Sequencing by High-Throughput Next-Generation Sequencing

Whole exome sequencing was performed in five individuals (KMS-09, -18, -21, -23, and -61) who had no *MLL2* or *KDM6A* abnormality by HRM analysis. DNA was processed with a Sure-Select Human All Exon V4 kit (Agilent Technologies), sequenced on a HiSeq2000 (Illumina, Inc., San Diego, CA), and analyzed as previously described [Tsurusaki et al., 2013]. Variants in *MLL2* and *KDM6A* were confirmed by Sanger sequencing.

X-Inactivation Assay

X-inactivation analysis was performed as described [Allen et al., 1992] with slight modification. Briefly, genomic DNA (500 ng) was digested with two methylation-sensitive restriction enzymes, *HpaII* and *HhaI* (New England Biolabs, Beverly, MA), and purified by phenol/chloroform extraction and ethanol precipitation. Digested and undigested DNA samples (10 ng) were separately amplified for the (CAG) n polymorphism at the androgen receptor locus. The forward primer was labeled with 5' FAM dye. PCR products were analyzed on an ABI 3500xl Genetic Analyzer using GeneMapper Software Version 4.1 (Applied Biosystems). The assay was independently performed twice.

cDNA Sequencing

Total RNA was extracted from a lymphoblastoid cell line established from KMS-81 (c.1909_1912del in *KDM6A*) using an RNeasy Plus mini kit (Qiagen, Hilden, Germany) with and without cycloheximide treatment (30 μ g/ml) for 4 hr before cell collection. Reverse transcription (RT) was performed using a Superscript III First-Strand synthesis system for RT-PCR (Life Technologies, Inc.). As the mutation was located in exon 17, the region from the exon 15/16 boundary to the exon 17/18 boundary of *KDM6A* was amplified using cDNA-specific primer pairs (sequences available on request) and sequenced by the Sanger method.

Statistical Analysis

The frequencies of clinical features in the two groups were compared by Fisher's exact test. A difference was considered statistically

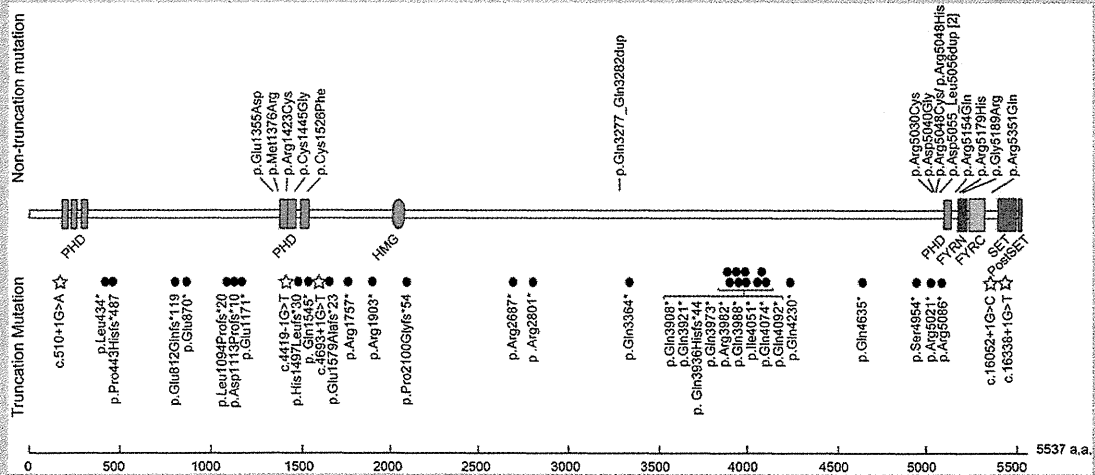


FIG. 1. *MLL2* mutations in patients with Kabuki syndrome. Upper: Non-truncating-type mutations. Middle: *MLL2* protein structure with functional domains. The protein contains seven plant homeodomains (PHD), one high-mobility group (HMG) domain, a Phe-Tyr-rich N-terminal (FYRN) domain, a Phe-Tyr-rich C-terminal (FYRC) domain, a SET (Suvar3-9, Enhancer-of-zeste, Trithorax) domain, and a PostSET domain. These functional domains were based on a prediction by SMART (<http://smart.embl-heidelberg.de/>) and the UniProtKB database (<http://www.uniprot.org/uniprot/O14686>). Lower: Truncating-type mutations. Black circles denote nonsense mutations or frameshift mutations. Stars represent splice-site mutations.

significant if $P < 0.05$. Correction for multiple testing was not applied.

RESULTS

Overall Mutation Detection Rates

Pathogenic mutations in *MLL2* and *KDM6A* were found in 50 (61.7%) and five (6.2%) of the 81 patients with KS, respectively (Figs. 1 and 2, Tables I and II). Of the 50 *MLL2* mutations, 35 (70.0%) were predicted to be protein truncating-type and 15 (30.0%) were predicted to be non-truncating-type. Interestingly, non-truncating mutations were mostly localized in or adjacent to the functional domains, while truncating mutations were scattered

throughout the entire coding region (Fig. 1). Fifteen of the *MLL2* mutations have been previously reported (Table I). Three novel variants (not included in the 50 mutations) were considered non-pathogenic (Supplemental Table I). Variant c.10942C > G in patient KMS-22 was predicted to be benign by Polyphen-2 and MutationTaster, c.8813C > T in patient KMS-62 was inherited from an unaffected father, and c.4065A > T in KMS-75 was found heterozygously in our 977 in-house controls. An in-frame duplication in patients KMS-40 and KMS-62, which predicted p.Asp5055_Leu5056dup, was predicted to be polymorphic by MutationTaster, but was previously reported as a pathogenic mutation [Micale et al., 2011]. In addition, the other in-frame mutation in KMS-02 was also predicted to be polymorphic. Unfortunately, parental samples were unavailable for these individuals, except for the mother of patient KMS-62, who did not have this mutation; thus, the de novo status remains unclear. Of the five *KDM6A* mutations including three mutations reported previously [Miyake et al., 2013], four were truncating-type and one was an in-frame deletion located within the Jumonji C domain (Fig. 2).

Clinical Comparison Between the Mutation-Positive and -Negative Groups

We compared the clinical features of the *MLL2* or *KDM6A* mutation-positive and -negative groups (Supplemental Table II). Long palpebral fissures were observed in almost all patients. Cleft lip/palate was more frequently observed in the mutation-positive group ($P = 0.0197$). Interestingly, developmental delay and intellectual disability were observed in all individuals with mutations but were unobserved in some mutation-negative cases ($P = 0.0314$

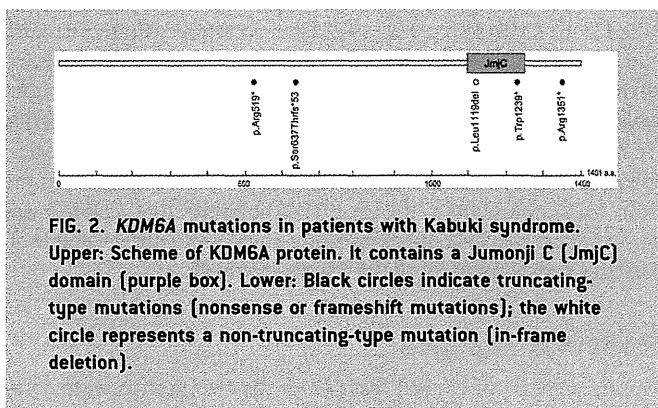


FIG. 2. *KDM6A* mutations in patients with Kabuki syndrome. Upper: Scheme of *KDM6A* protein. It contains a Jumonji C (JmjC) domain (purple box). Lower: Black circles indicate truncating-type mutations (nonsense or frameshift mutations); the white circle represents a non-truncating-type mutation (in-frame deletion).

TABLE I. *MLL2* and *KDM6A* Mutations in Patients With KS

Patient ID	Method	Mutation	Predicted amino acid change	De novo	Remarks ^a
Patients with <i>MLL2</i> mutations					
KMS-02	H	c.9831_9848dup	p.Gln3277_Gln3282dup	Unknown	Novel
KMS-08	H	c.12688C > T	p.Gln4230*	Yes	Hannibal et al. [2011]
KMS-13	H	c.2433_2434insCA	p.Glu812Glnfs*119	Yes	Novel
KMS-14	H	c.11806_11807dup	p.Gln3936Hisfs*44	Yes	Novel
KMS-15	H	c.15119A > G	p.Asp5040Gly	Yes	Novel
KMS-17	H	c.5707C > T	p.Arg1903*	Yes	Novel
KMS-18	W	c.12151delA	p.Ile4051*	Yes	Novel
KMS-20	H	c.1300delC	p.Leu434*	Unknown	Novel
KMS-21	W	c.3326_3336dup	p.Asp1113Profs*10	Unknown	Novel
KMS-22	H	c.4127T > G	p.Met1376Arg	Unknown	Novel
KMS-23	W	c.15461G > A	p.Arg5154Gln	Unknown	Li et al. [2011]
KMS-24	H	c.2608 G > T	p.Glu870*	Unknown	Novel
KMS-25	H	c.11917C > T	p.Gln3973*	Unknown	Novel
KMS-27	H	c.15142C > T	p.Arg5048Cys	Yes	Hannibal et al. [2011], Makrythanasis et al. [2013]
KMS-28	H	c.14861C > A	p.Ser4954*	Unknown	Novel
KMS-29	H	c.4419-1G > T	splice site	Unknown	Novel
KMS-30	H	c.4633C > T	p.Gln1545*	Unknown	Novel
KMS-32	H	c.8059C > T	p.Arg2687*	Unknown	Banka et al. [2012b]
KMS-33	T	c.11962C > T	p.Gln3988*	Unknown	Novel
KMS-36	H	c.4736_4737delAG	p.Glu1579Alafs*23	Unknown	Novel
KMS-38	H	c.15143G > A	p.Arg5048His	Unknown	Makrythanasis et al. [2013]
KMS-40	T	c.15163_15168dup	p.Asp5055_Leu5056dup	Unknown	Micale et al. [2011]
KMS-41	H	c.1328delC	p.Pro443Hisfs*48?	Yes	Ng et al. [2010]
KMS-42	H	c.16052G > A	p.Arg5351Gln	Yes	Novel
KMS-43	H	c.510 + 1G > A	splice site	Unknown	Novel
KMS-49	T	c.15565G > A	p.Gly5189Arg	Unknown	Novel
KMS-51 ^b	H	c.6297_6298delAC	p.Pro2100Glyfs*54	Yes	Novel
KMS-52	H	c.4693 + 1G > T	splice site	Yes	Novel
KMS-53	H	c.10090C > T	p.Gln3364*	Yes	Novel
KMS-54	T	c.8401C > T	p.Arg2801*	Yes	Novel
KMS-56	H	c.15536G > A	p.Arg5179His	Yes	Ng et al. [2010]
KMS-58	H	c.4333T > G	p.Cys1445Gly	Yes	Novel
KMS-59	H	c.15256C > T	p.Arg5086*	Yes	Banka et al. [2012b]
KMS-60	H	c.11761C > T	p.Gln3921*	Unknown	Novel
KMS-61 ^c	W	c.5269C > T	p.Arg1757*	Yes	Novel
KMS-62	H	c.15163_15168dup	p.Asp5055_Leu5056dup	Unknown ^f	Micale et al. [2011]
KMS-63	T	c.4577G > T	p.Cys1526Phe	Yes	Novel
KMS-69	H	c.11944C > T	p.Arg3982*	Yes	Paulussen et al. [2011]
KMS-70	T	c.13903C > T	p.Gln4635*	Yes	Novel
KMS-71 ^d	T	c.12220C > T	p.Gln4074*	Unknown	Novel
KMS-72	T	c.15061C > T	p.Arg5021*	Yes	Banka et al. [2012b]
KMS-73	T	c.12274C > T	p.Gln4092*	Unknown	Micale et al. [2011]
KMS-76	T	c.4490_4491delAC	p.His1497Leufs*30	Yes	Novel
KMS-78	T	c.16338 + 1G > T	splice site	Yes	Novel
KMS-80	T	c.15088C > T	p.Arg5030Cys	Unknown	Makrythanasis et al. [2013]
KMS-82	T	c.3511G > T	p.Glu1171*	Yes	Novel
KMS-85	T	c.11722C > T	p.Gln3908*	Yes	Paulussen et al. [2011]
KMS-87	T	c.3281_3282delTTC	p.Leu1094Profs*20	Yes	Novel
KMS-88	T	c.16052 + 1G > C	splice site	Unknown	Novel
KMS-91	T	c.4267C > T	p.Arg1423Cys	Unknown	Novel
Patients with <i>KDM6A</i> mutations					
KMS-31 ^e	H	c.3717G > A	p.Trp1239*	Unknown	Miyake et al. [2013]
KMS-37 ^e	H	c.1555C > T	p.Arg519*	Unknown	Miyake et al. [2013]
KMS-65 ^e	H	c.3354_3356delTCT	p.Leu1119del	Yes	Miyake et al. [2013]
KMS-81	T	c.1909_1912delTCTA	p.Ser637Thrfs*53	Yes	Novel
KMS-83	T	c.4051C > T	p.Arg1351*	Unknown	Novel

H, high-resolution melting analysis/Sanger sequencing; T, targeted resequencing; W, whole exome sequencing. RefSeq NM_003482.3 for *MLL2* and RefSeq NM_021140.2 for *KDM6A* were used as reference sequences.

^aReferences are listed when the same mutation has been reported previously.

^bThis patient was reported as proband 1 by Tekin et al. [2006].

^cThe detailed clinical features of this patient were reported by Ito et al. [2013], because of her hypothalamic-pituitary complications.

^dThe clinical course of this patient, particularly the idiopathic thrombocytopenic purpura, was reported by Torii et al. [2009].

^eThese patients have been reported in our previous study [Miyake et al., 2013].

^fPatient KMS-62: no mutation in the mother.

TABLE II. *MLL2* Non-Truncating-Type Mutations in Patients With KS

Amino acid change ^a	Patient ID	Domain	Polyphen-2 (score)	MutationTaster
p.Met1376Arg	KMS-22	—	Probably damaging (0.915)	Polymorphism
p.Arg1423Cys	KMS-91	PHD	Probably damaging (1.000)	Disease causing
p.Cys1445Gly	KMS-58	PHD	Probably damaging (1.000)	Disease causing
p.Cys1526Phe	KMS-63	PHD	Probably damaging (0.999)	Disease causing
p.Gln3277_Gln3282dup	KMS-02	—	NA	Polymorphism
p.Arg5030Cys	KMS-80	—	Probably damaging (1.000)	Disease causing
p.Asp5040Gly	KMS-15	—	Probably damaging (1.000)	Disease causing
p.Arg5048Cys	KMS-27	—	Probably damaging (1.000)	Disease causing
p.Arg5048His	KMS-38	—	Probably damaging (1.000)	Disease causing
p.Asp5055_Leu5056dup	KMS-40, 62	—	NA	Polymorphism
p.Arg5154Gln	KMS-23	—	Probably damaging (1.000)	Disease causing
p.Arg5179His	KMS-56	FYRN	Possibly damaging (0.840)	Disease causing
p.Gly5189Arg	KMS-49	FYRN	Probably damaging (1.000)	Disease causing
p.Arg5351Gln	KMS-42	—	Probably damaging (1.000)	Disease causing

^aThe nucleotide mutation nomenclature for these predicted protein mutations are included in Table I.

and $P = 0.1778$, respectively). Blue sclera, lower lip pits, spine/rib abnormality, hip joint dislocation, umbilical hernia, kidney dysfunction, cryptorchidism, liver abnormality, spleen abnormality, premature thelarche, neonatal hyperbilirubinemia, and anemia were observed only in the mutation-positive group.

Clinical Comparison of the *MLL2*-Mutated and *KDM6A*-Mutated Groups

We compared the clinical features between the *MLL2*-mutated and *KDM6A*-mutated groups (Figs. 3–5, Supplemental Table III). High arched eyebrows, short fifth fingers, and hypotonia in infancy were more frequent in individuals with *MLL2* mutations than in individuals with *KDM6A* mutations ($P = 0.0364$, 0.0039 , and 0.0283 , respectively). Short stature was more frequent in individuals with *KDM6A* mutations ($P = 0.0485$). Although not statistically significant, postnatal growth retardation was observed in all individuals with *KDM6A* mutations, whereas this was observed in only half of the individuals with *MLL2* mutations.

Clinical Comparison of Individuals With a *MLL2* Truncating-Type and Non-Truncating-Type Mutation

Most clinical features were observed at a similar ratio in both groups (Supplemental Table IV), except for prominent ears and hypotonia, which were more frequently observed in the truncating-type group than in the non-truncating-type group ($P = 0.0339$ and $P = 0.0248$, respectively). However, the facial appearance of individuals in the truncating-type group was more typical, based on the ten originally reported patients with KS [Kuroki et al., 1981; Nii-kawa et al., 1981], than that in the non-truncating-type group (Figs. 3 and 4). Except for patient KMS-58, the facial appearance of patients with a non-truncating-type mutation was rather less typical. It should be noted that these patients had thick eyebrows (not present in patient KMS-56). Furthermore, ectropion of the

lower eyelid, depressed nasal tip, short columella, and prominent ears all seemed less obvious in the individuals with a non-truncating-type mutation.

X-Inactivation Pattern in Female Patients With a *KDM6A* Mutation

A *KDM6A* mutation was identified in two females (KMS-65 and KMS-81). Individual KMS-65 (c.3354_3356del, which predicts p.Leu119del) showed a random X-inactivation pattern [Miyake et al., 2013], while individual KMS-81 with a frame-shift mutation showed marked skewing (98:2; Supplemental Fig. 1A). By RT-PCR using mRNA derived from a lymphoblastoid cell line from patient KMS-81, we confirmed that both the mutated and normal alleles were transcribed at similar levels when nonsense-mediated mRNA decay (NMD) was inhibited by cycloheximide treatment (Supplemental Fig. 1B). In untreated cells, or cells treated with dimethylsulfoxide (negative control), the mutant allele was transcribed at a lower level than the wild-type allele, indicating that NMD partially eliminated the mutant.

Exome Sequencing

Among the five patients who were also analyzed by whole exome sequencing, mutations were identified and later confirmed by Sanger sequencing in four (Table I). Fragments with an 11 base-pair insertion (c.3326_3336dup) of *MLL2* in patient KMS-21 could not be amplified by Ex Taq, but could be amplified by LA Taq with LA buffer and confirmed by Sanger sequencing. Three other mutations were missed by HRM analysis (Table I).

DISCUSSION

We identified 50 *MLL2* and five *KDM6A* mutations among 81 patients with KS and add to the 246 *MLL2* mutations described in patients with KS [Ng et al., 2010; Hannibal et al., 2011; Li et al., 2011; Micale et al., 2011; Paulussen et al., 2011; Banka et al., 2012a,b;