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Kleine-Levin syndrome is associated with *LMOD3* variants

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Abstract

Kleine-Levin syndrome (KLS) is a rare periodic hypersomnia with associated behavioural abnormalities but with often favourable prognosis. There is excess risk of KLS in first-degree relatives, suggesting a strong genetic contribution. So far, no mutation is identified in KLS and comprehensive genetic analysis of affected individuals is lacking. Here we performed whole genome single-nucleotide polymorphism (SNP) genotyping and exome sequencing in a large family with seven affected members. The identified gene with a mutation was resequenced in 38 sporadic KLS patients and the expression of the gene product was mapped in the mouse brain. Linkage analysis mapped the disease locus to chromosome 3 and exome analysis identified a heterozygous missense variant in *LMOD3* (p.E142D) in the linkage interval. The variant was found to segregate in all affected and one presumably unaffected member of the family. Resequencing *LMOD3* in 38 other KLS patients and their families revealed three other low frequency or rare missense variants in seven cases that were inherited with incomplete penetrance. LMOD3 is expressed in the brain and colocalized with major structures involved in the regulation of vigilance states. LMOD proteins are structural proteins and seem to be developmentally regulated. Our findings suggest that KLS might be a structural/neurodevelopmental brain disease.

*These authors contributed equally to this work.

KEYWORDS
exome, hypocretin, lateral hypothalamus, Leiomodin 3, linkage, periodic hypersomnia

1 | INTRODUCTION

Kleine-Levin syndrome (KLS) is a rare, relapsing-remitting, debilitating sleep disorder affecting about one to five in 2 million individuals, usually adolescents (Arnulf, 2015). KLS patients experience periods of alternating normality and hypersomnia lasting one to a few weeks accompanied by cognitive, behavioural and psychiatric disturbances (Arnulf, 2015). The prognosis of KLS is often favourable, with less frequent and shorter episodes throughout the disease process before resolving in most patients (Billiard, Jaussent, Dauvilliers, & Besset, 2011). KLS is thought to have a genetic component because first-degree relatives of affected individuals have an 800–4,000-fold increased risk of developing KLS (Arnulf, Rico, & Mignot, 2012), multiplex families with KLS exist (BaHammam, Gadelrab, & Hamam, 2008; Katz & Ropper, 2002; Poppe et al., 2003; Rocamora, Gil-Nagel, Franch, & Vela-Bueno, 2010), including affected monozygotic twins (Peraita-Adrados, Vicario, Tafti, Garcia De Leon, & Billiard, 2012; Ueno, Fukuhara, Ikegami, Ohishi, & Kume, 2012), and there is a slightly higher prevalence in Ashkenazi Jews, suggesting a founder effect (Arnulf et al., 2008). KLS is also reported to be associated with HLA-DQB1*02 (Dauvilliers et al., 2002), but this association was difficult to replicate (Arnulf et al., 2008; Lavault et al., 2015; Nguyen et al., 2016). A recent genome-wide association study found a variant in *TRANK1* associated with KLS (Hillary et al., 2017). So far no mutation is identified in KLS and comprehensive genetic analysis of affected individuals is lacking (Raizen & Wu, 2011).

Identifying recurrent mutations in KLS families is expected to provide important insights into KLS pathogenesis and the genetic basis of sporadic cases, because there are no marked phenotypic, HLA or karyotypic differences between familial and sporadic cases beyond minor differences in severity (Al Suwayri, 2016; Nguyen et al., 2016). We therefore performed linkage analysis and exome sequencing in a large KLS family with seven affected members and identified a mutation in *LMOD3*. We further validate this finding by resequencing LMOD3 in 38 other KLS patients and mapped its expression in the mouse brain.

2 | METHODS

2.1 | Patients

2.1.1 | Saudi Arabian KLS family and KLS diagnostic criteria

Seven surviving patients in a consanguineous family (Figure 1) were diagnosed with KLS at the Sleep Center, King Saud University (BaHammam et al., 2008). Participants provided written informed consent and the institutional review board (IRB) of King Saud University approved the study protocol.

All included patients met the criteria for KLS according to the International Classification of Sleep Disorders. All participants underwent full history and examination to confirm the diagnosis of KLS and to rule out concurrent disease between June 2013 and July 2014. Eligible participants also completed the Stanford KLS questionnaire in English. Anxiety and depression assessments were based on the Hospital Anxiety and Depression Scale (Zigmond & Snaith, 1983), and attitudes to eating were assessed using the EAT-26 eating attitudes test (Garner, Olmsted, Bohr, & Garfinkel, 1982).

2.1.2 | Other KLS patients

Thirty-eight European KLS patients and their relatives were also investigated. This population included the 30 patients reported by Dauvilliers et al. (2002), a patient reported by Haba-Rubio et al. (2012) and seven new patients. Thirty-seven cases were sporadic and one was familial (a case with a mother who also suffered from KLS in her adolescence). In 24 cases both parents were available, in five cases only one parent was available, and the nine others were index cases.

2.2 | Linkage analysis

All participants underwent venipuncture and genomic DNA was extracted using standard techniques. Seventeen subjects from the Saudi Arabian family (six affected and 11 unaffected or unknown status; Figure 1) were genotyped using the HumanCytoSNP-12 v2.1 Bead Chip (Illumina, San Diego, CA, USA). After filtering and removing non-informative single-nucleotide polymorphisms (SNPs), 218,361 SNP genotypes were analysed with Superlink-Online SNP version 1.0 (https://cbl-hap.cs.technion.ac.il/superlink-snp/) (Silberstein et al., 2006). Although there are two consanguineous marriages in the family, the pedigree and segregation analyses favoured an autosomal dominant mode of inheritance. Thus, a dominant model with a mutant allele frequency of 0.001 and penetrance of 0.9 was used for linkage analysis.

2.3 | Exome sequencing

Exomes were captured using Agilent SureSelect Human All Exon v4 enrichment kits (Agilent Technologies, Santa Clara, CA, USA) and sequenced on the Illumina HiSeq 2500 platform (Illumina, San Diego, CA, USA). Variant calling and quality filtering were performed per sample using GATK UnifiedGenotyper and GATK Variant Quality

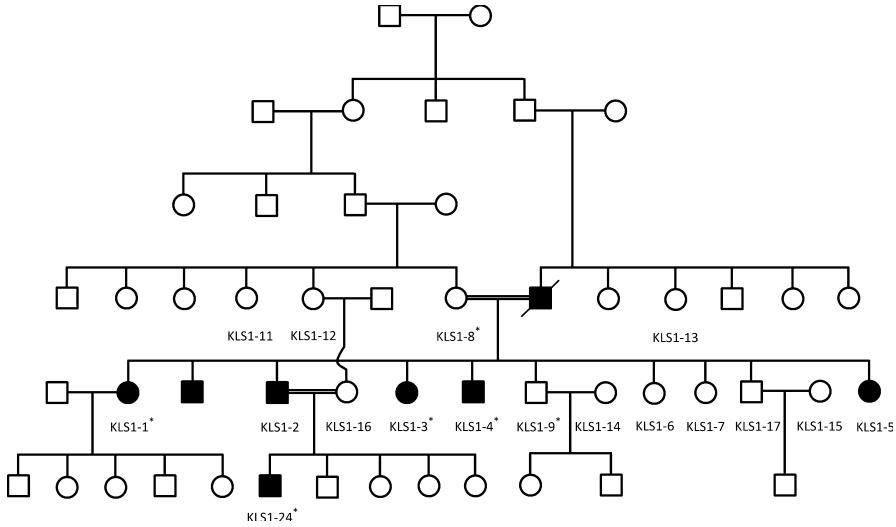

FIGURE 1 Pedigree of the Saudi Arabian family. Circles indicate women and squares men. Black filled symbols indicate affected and clear symbols indicate unaffected family members. Numbers indicate family members for whom DNA was available for analysis. *Indicates family members that were exome sequenced

Score Recalibration and were annotated with Annovar. Both a dominant and a recessive model were tested but the recessive model did not result in any potential homozygous mutation in the four affected and heterozygous mutation in the two non-affected family members.

2.4 | LMOD3 resequencing

The coding region of *LMOD3*, except for the last exon encoding the 8 terminal amino acids, was sequenced in both directions by Sanger sequencing. All cases and their relatives were sequenced with primers: Exon1 forward: 5′- TGCTCAGCAAACCACTGAGG-3′, reverse: 5′-CAGAGAGACCTAACAGCCCA-3′, Exon2 forward1: 5′-ATCTCCACTAGCTGATGCTCC-3′ Exon2 reverse1: 5′-TGACCCAACATGTGCCTCTG-3′, Exon2 forward2: 5′- GGCCATCATGAGGTGTCTCC-3′, Exon2 reverse2: 5′- CTCAGTCACCATTTCTCCCTCC-3′.

2.5 | LMOD3 immunohistofluorescence

C57BL76 J mice at the age of 8–11 weeks were deeply anaesthetized with sodium pentobarbital (150 mg/ml diluted at 1:14, i.p. in a volume of 10 ml/kg bodyweight) and perfused transcardially with heparin/phosphate buffered saline (PBS), followed by a 10-min fixation with 4% paraformaldehyde, pH 7.2, in PBS at the speed of 16 ml/min. Brains were quickly removed and post-fixed in the same fixative solution overnight at 4°C, immersed in 15% sucrose 1 hr at 4°C and 30% sucrose overnight at 4°C. Brains were frozen in cold isopentane for 10 min and stored at –80C until used. Cryosections (20 μm thin) were mounted on SuperFrost-Plus glass slides. A heating-induced water-bath antigen-retrieval technique was applied (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) before blocking in 1 × PBS + 0.05% Tween 20 + 1% bovine serum albumin (BSA) + 10% normal donkey serum for 1 hr at room temperature. Primary antibodies were applied in 1 × PBS + 0.05% Tween 20 + 5% normal donkey serum and incubated overnight at 4°C. Antibodies were: LMOD3 from rabbit (Proteintech, 14948–1-AP, 1:100), tyrosine hydroxylase (TH) from mouse (Incstar, Cat. 22941, 1:5000), hypocretin-1/orexin A (C-19) (HCRT) from goat (Santa Cruz Biotechnology, SC-8070, 1:500), tryptophan hydroxylase (TPH) from mouse (Sigma, T0678, 1:1,000) and histidine decarboxylase (HDC) from rabbit (Progen, Cat. 16,045, 1:500). Secondary antibodies were donkey IgGs coupled to Alexa dyes used at 1:500 for 2 hr at room temperature.

3 | RESULTS

The Saudi Arabian family included eight affected members (Figure 1) and had been previously reported (BaHammam et al., 2008). At the time of investigations, seven affected members were alive and all except one agreed to participate in the study (Table 1). Briefly, the six affected family members included were aged between 14 and 43 years and all had experienced their first KLS episode in adolescence (range 12–16 years, average 13.8 years, standard deviation [*SD*] 1.5 years). Three were males and three were female. Of note, five out of six of the family also had a diagnosis of ankylosing spondylitis and reported mild to moderate depressive symptoms, and four of six had atopic syndrome. Two of the three women described first menarche immediately prior to the first episode, and one boy experienced fever and sore throat immediately prior to presentation. All individuals described typical symptoms of KLS, with episodes lasting days to several weeks, and hypersomnia, hyperphagia and hypersexuality were common.

3.1 | Mapping a KLS locus on chromosome 3

Multipoint linkage analysis of the six affected and 11 unaffected or unknown-status individuals (Figure 1) indicated a single peak with an logarithm of odds (LOD) score of 2.41 on chromosome 3 (Figure 2) between rs481319 (position Chr3:62,728,945) and rs7636827

TABLE 1 Demographic and clinical history of the Saudi Arabian family with KLS

Characteristics	*N*, % or mean ± *SD*
Number of subjects	6
Age at interview, years	28.7 ± 11.8
Male sex, %	50.0
BMI	24.0 ± 4.8
Medical history, %	
Birth difficulties	16.7
Allergy (asthma, rhinitis)	66.7
Family history, %	
Kleine-Levin syndrome	100
Depression	83.3
Ankylosing spondylitis	83.3
Symptoms before first episode	
First menarche	33.3
Age at disease onset, years	13.3 ± 1.5
Disease duration, years	14.8 ± 11.9
First episode duration, days	11.5 ± 9.7
Mean episode duration, days	14.0 ± 20.6
Sleep symptoms during symptomatic periods	
Hypersomnia, %	100
Time in episode, hr/24 hr	12.7 ± 3.1
Cognitive symptoms, %	
Cognitive impairment	100
Impaired speech	100
Confusion	100
Altered perception/derealization	100
Apathy	100
Eating behaviour disturbance, %	
Megaphagia	83.3
Decreased appetite	16.7
Sexual disturbances, %	66.7
Hypersexuality or disinhibition	16.7
Other psychiatric symptoms, %	
Hallucinations/delusions	
Split body/mind, feeling of disembodiment	100
Agitation/excitation	66.7
Anxiety	83.3
Depression	83.3
Meningeal symptoms, %	16.7
Photophobia	16.7
Hyperacusia	100
Headache	50.0
Other symptoms, %	
Lost sense of time	66.7
Lost space perception	83.3
Difficulty concentrating	100
Impaired motor skills	83.3
Difficulty reading	100
Memory disturbance	100
Difficulty making decision	100
Difficulty with two simultaneous tasks	66.7
Altered perception of environment	100
Post-episode symptoms, %	
Incomplete recollection of episodes	83.3

BMI: body mass index; *SD*: standard deviation.

(position Chr3:73,101,899). No other suggestive loci were found (LOD score > 1.9 (Lander & Kruglyak, 1995), Figure 2). Haplotype analysis revealed a shared haplotype between all affected subjects and one presumably unaffected (KLS1–17) subject. The same analysis with a recessive model did not indicate any loci except a few single SNPs with LOD score > 1.9 (chromosomes 1, 2 and 7).

3.2 | Exome sequencing identified a missense variant in *LMOD3*

Six members of the family that included four affected and two unaffected (indicated by * in Figure 1) participants, were exome sequenced. After filtering, four potential missense mutations were detected (Figure 2): *PCDH9* (protocadherin 9, Ch13: 66,302,834-67,230,445), *COL6A2* (collagen type VI alpha 2 chain, Ch21: 46,098,097-46,132,849), *PCNT* (pericentrin, Ch21: 46,324,122-46,445,769) and *LMOD3* (leiomodin 3, Ch3: 69,106,872-69,123,032). Of these, only *LMOD3* mapped to the linkage region on chromosome 3 (Figure 2). We nevertheless resequenced all coding exons of the four genes in all 17 available members of the family: the *PCDH9* variant was excluded because it was absent in two affected individuals but present in two unaffected individuals; the *COL6A2* and *PCNT* variants were not considered candidates because of their absence in one affected individual and presence in one unaffected subject. Only the variant (p.E142D) in *LMOD3* was present in all affected subjects. In accordance with the linkage analysis, KLS1-17 also carries this mutation. No homozygous or compound heterozygous mutation compatible with a recessive model was identified (including in the three regions with suggestive linkage on chromosomes 1, 2 and 7). To make sure that no other sequence variants within the linkage region were missed, we also looked for any copy-number variation but found none. Also, we identified low-coverage regions (<10x) with the GATK tool FindCoveredIntervals and the resulting intervals from each sample were merged, visualized in IGV and analyzed with GATK DiagnoseTargets. The only protein coding region with low coverage in all four affected subjects was the first 50 amino-acid of GXYLT. These results strongly suggest that the only variant in the linkage region is p.E142D in *LMOD3*.

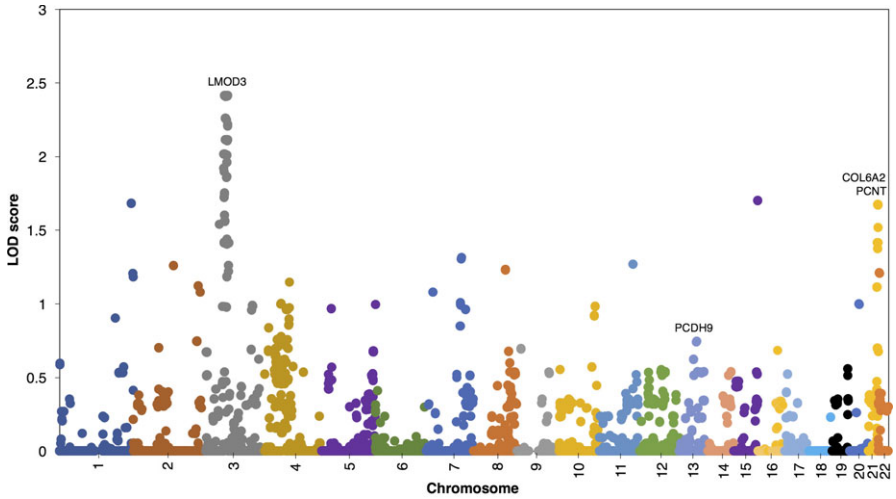

FIGURE 2 Manhathan plot showing the multipoint linkage analysis LOD scores. LOD scores are plotted against SNPs covering each chromosome. Genes with potential mutations found in the exome sequencing are indicated

3.3 | Resequencing LMOD3 found additional variants

We next sequenced *LMOD3* in 38 KLS patients of European origin. Three new missense variants in addition to the p.E142D were found in seven KLS patients (Table 2). The original p.E142D was found in a sporadic KLS case. One variant (p.R83H) was found in three independent sporadic KLS patients. The two other variants (p.K282E and p.P552H) were found in three independent cases. In one of the sporadic cases a compound p.R83H/p.P552H mutation was found. Variants p.R83H and p.P552H are predicted to be damaging. In six cases with mutation, DNA from both parents was available and in all the mutation was transmitted (three times from the unaffected father and three times from the unaffected mother), indicating incomplete penetrance. Overall, by screening our replication cohort, three low-frequency (MAF < 0.05) and two rare (MAF < 0.01) variants were found in seven independent KLS patients (Table 2). We have compared these variants with their frequencies reported in ExAC (Exome Aggregation Consortium) by Fisher's exact test and found that p.E142D ($p = 0.03$) and p.K282E ($p < 10^{-6}$) were significantly increased. Finally, the familial p.E142D variant was also found in two other sporadic KLS patients by the International Genetic Study of Kleine-Levin Syndrome (University College of London, London, UK) as part of an ongoing exome sequencing project including 45 KLS cases (combined $p < 0.004$). Also, a new mutation, p.G293D, was found in two other sporadic cases by the same study group. p.G293D has a low frequency (0.0161) and is predicted to be pathogenic. Patients with these variants were not significantly different from others in terms of age at onset or severity (number of hypersomnia episodes).

3.4 | LMOD3 is expressed in key brain structures

Because the expression of LMOD3 protein is not reported in the brain, we mapped its expression in serial sections from C56BL/6J mice. LMOD3 was found to be extensively expressed in brain structures, including the cortex, hypothalamus, hippocampus, mesopontine and brainstem. Double immunofluorescence staining indicated that nearly all hypocretin (Figure 3 a–d), dopamine (Figure 3 i–h), serotonin (Figure 3 m–p), noradrenaline (Figure 3 q–t) and most

TABLE 2 *LMOD* mutations found in sporadic KLS patients

Patient	Position[a]	SNP	Mutation[b]	Amino acid[c]	Inherited	MAF[d]
1 M	69122139	rs35740823	c.G248A	p.R83H	Yes	0.0439
2 M	69122139	rs35740823	c.G248A	p.R83H	Yes	0.0439
3F	69122139	rs35740823	c.G248A	p.R83H	Yes	0.0439
3F	69119929	rs111848977	c.A426C	p.E142D	Yes	0.0036
4F	69118700	rs145387235	c.C1655A	p.P552H	Yes	0.0183
5 M	69118700	rs145387235	c.C1655A	p.P552H	Yes	0.0183
6F	69119510	—	c.A1054G	p.K282E	Yes	—

[a]Position on Chr3, reference genome GRCh38.p10.
[b]Nucleotide, reference sequence NM_198271.4.
[c]Amino acid, reference sequence NP_001291347.1.
[d]MAF = minor allele frequency in non-Finnish Europeans from ExAC.

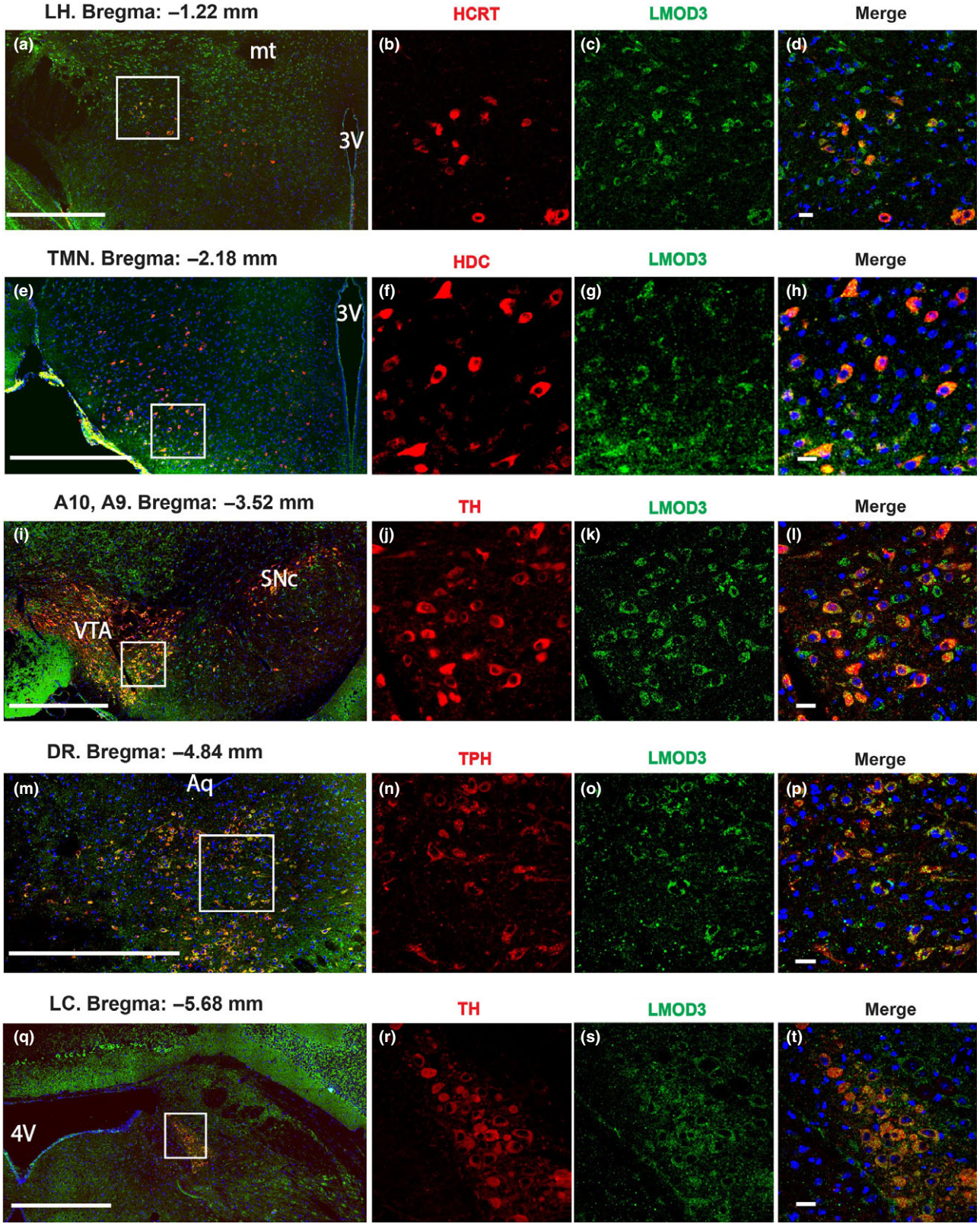

FIGURE 3 Expression of LMOD3 in the mouse brain. (a), (e), (i), (m), (q) Coronal mouse brain sections at the level of the lateral hypothalamus (LH), tuberomammillary nucleus (TMN), ventral tegmental area (VTA) and substantia nigra pars compacta (SNc), dorsal raphé (DR) and locus coeruleus (LC) at the indicated stereotaxic level with reference to Bregma. Regions denoted by white squares are shown at higher magnification in (b), (c), (d), (f), (g), (h), (j), (k), (l), (n), (o), (p), (r), (s) and (t). HCRT, hypocretin; LMOD3, leiomodin 3; HDC, histidine decarboxylase; TH, tyrosine hydroxylase; TPH, tryptophan hydroxylase; mt, mammillary tract; 3 V, third ventricle; Aq, aqueduct; 4 V, fourth ventricle. Blue colour indicates 4′,6-diamidino-2-phenylindole (DAPI) fluorescence of cell nuclei staining. Scale bars: 500 μm at low and 20 μm at high magnification

histamine (Figure 3 i–h) producing neurons, essential for the regulation of wakefulness, were LMOD3 positive.

4 | DISCUSSION

Here we report molecular analyses of a unique and large KLS family. Linkage analysis and exome sequencing identified a KLS-related low-frequency variant in *LMOD3*. Little is known about the function of *LMOD3*, although homozygous and compound heterozygous mutations in *LMOD3* were described in patients with nemaline myopathy, a congenital myopathy characterized by muscle weakness and protein inclusions in skeletal myofibers (Yuen et al., 2014).

The p.E142D variant found in our family and three sporadic cases is localized in the Glu-rich domain of *LMOD3*, a domain of unknown function. None of the variants reported here were found in patients with nemaline myopathy, which is characterized by mutations in the actin-binding domain (Yuen et al., 2014), although the p.K282E variant found in one of our sporadic KLS patients is also within one of the actin-binding domains without any sign of myopathy. Note that p.K282E is a new mutation never reported before. Whether the variants found here are causally (functionally) linked to KLS cannot be demonstrated at this stage because of the lack of a functional model (in a neuron-based system), although three out of the five variants are predicted by in-silico analysis to be pathogenic. Given that seven out of our 38 cases (18.4%) carried an *LMOD3* missense variant and four other sporadic cases (8.9%) from the UK exome sequencing project also carry two *LMOD3* variants, and our brain localization of LMOD3 protein indicated colocalization with all major waking structures, we believe that variants found here might be implicated in KLS pathophysiology. The variants reported here had never been associated with another disease. As KLS is a rare disease with an estimated prevalence of less than five in 2 million individuals, we cannot exclude the implication of secondary (modifying) genetic or environmental factors in disease onset.

LMOD3 is a structural protein with an unknown role in the nervous system. LMOD1 is extensively expressed in the brain (the cortex, hippocampus and cerebellum) and might be a target of autoantibodies in nodding syndrome, a syndrome characterized by seizures (Johnson et al., 2017). The expression of LMOD2 is confined to the thalamus and is strongly upregulated by phencyclidine, a schizophrenomimetic drug, in rats (Takebayashi, Yamamoto, Umino, & Nishikawa, 2009). LMOD proteins are not simply restricted to the heart and skeletal muscle and might have structural and or developmental functions in critical regions of the central nervous system implicated in KLS. Several imaging studies in KLS patients reported abnormal perfusion patterns in the hypothalamus that might corroborate the hypothesis that KLS represents a disorder of diencephalic or hypothalamic function (Carpenter, Yassa, & Ochs, 1982; Critchley, 1962; Gadoth, Kesler, Vainstein, Peled, & Lavie, 2001; Malhotra, Das, Gupta, & Muralidharan, 1997; Takrani & Cronin, 1976). We found LMOD3 expression in the lateral hypothalamus, colocalized with hypocretin neurons. Potential implication of hypocretins (namely, a decreased cerebrospinal fluid [CSF] level during the hypersomnia period) was reported (Lopez, Barateau, Chenini, & Dauvilliers, 2015). A recent study in 44 Chinese KLS patients also reported that the CSF hypocretin-1 levels were 31% lower during relapse (Wang et al., 2016). In addition to hypocretin neurons, LMOD3 was also found to be expressed in the tuberomammillary locus, locus coeruleus, ventral tegmental area and substantia nigra, as well as in the raphe nucleus, all critically involved in the maintenance of wakefulness.

In summary, because KLS develops during adolescence with a favourable prognosis with age and our findings implicate LMOD3 variants, we propose that KLS might be a structural and/or developmental central nervous system disorder.

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CONFLICT OF INTEREST

All authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

MT and ASB conceived the study. SMAS, SB, SL, CP and SP performed the experiments. The other authors provided patients' and clinical data.

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