# Kleine-Levin syndrome is associated with *LMOD3* variants

Running head: LMOD3 association in KLS

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**SUMMARY**

Kleine-Levin syndrome (KLS) is a rare periodic hypersomnia with associated behavioral abnormalities but with often favorable 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 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 7 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.

***Keywords***: Periodic hypersomnia, Leiomodin 3, lateral hypothalamus, hypocretin, exome, linkage.

## INTRODUCTION

Kleine-Levin syndrome (KLS) is a rare, relapsing-remitting, debilitating sleep disorder affecting about 1 to 5 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, behavioral, and psychiatric disturbances (Arnulf, 2015). The prognosis of KLS is often favorable with less frequent and shorter episodes with the disease process before resolving in most patients (Billiard et al., 2011). KLS is thought to have a genetic component since first-degree relatives of affected individuals have an 800-4000-fold increased risk of developing KLS (Arnulf et al., 2012), multiplex families with KLS exist (BaHammam et al., 2008, Katz and Ropper, 2002, Poppe et al., 2003, Rocamora et al., 2010) including affected monozygotic twins (Peraita-Adrados et al., 2012, Ueno et al., 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 (Nguyen et al., 2016, Lavault et al., 2015, Arnulf et al., 2008). A recent genome-wide association study found a variant in *TRANK1* associated with KLS (Hillary RP, 2017). So far no mutation is identified in KLS and comprehensive genetic analysis of affected individuals is lacking (Raizen and Wu, 2011).

Identifying recurrent mutations in KLS families is expected to provide important insights into KLS pathogenesis and the genetic basis of sporadic cases, since 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.

## METHODS

**Patients:** *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 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 and Snaith, 1983), and eating attitudes were assessed using the EAT-26 eating attitudes test (Garner et al., 1982).

*Other KLS patients:* 38 European KLS patients and their relatives were also investigated. This population included the 30 patients reported by Dauvilliers et al., (Dauvilliers et al., 2002) a patient reported by Haba-Rubio et al., (Haba-Rubio et al., 2012), and 7 new patients. 37 cases were sporadic and one was familial (a case with the mother who also suffered from KLS in her adolescence). In 24 cases both parents were available, in 5 cases only one parent was available, and the 9 others were index cases.

**Linkage analysis:** All participants underwent venipuncture and genomic DNA was extracted using standard techniques. Seventeen subjects from the Saudi Arabian family (6 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 SNPs, 218,361 SNP genotypes were analysed with Superlink-Online SNP version 1.0 (http://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 favored 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.

**Exome sequencing:** Exomes were captured using Agilent SureSelect Human All Exon v4 enrichment kits (Agilent Technologies, Santa Clara, CA) and sequenced on the Illumina HiSeq 2500 platform (Illumina, San Diego, CA). Variant calling and quality filtering were performed per sample using GATK UnifiedGenotyper and GATK Variant Quality 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 4 affected and heterozygous in the 2 non-affected family members.

**LMOD3 resequencing:** The coding region of *LMOD3* except the last exon encoding the 8 terminal amino acids was sequenced on 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'.

**LMOD3 Immunohistofluorescence:** C57BL76J mice at the age of 8-11 weeks were deeply anesthetized with sodium pentobarbital (150 mg/ml diluted at 1:14, i.p. in a volume of 10 ml/kg body weight) and perfused transcardially with heparin/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 hour at 4°C and 30% sucrose overnight at 4°C. Brains were frozen in cold isopentane for 10 min and stored at -80°C until used. 20 µm thin cryosections 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 1xPBS+0.05% Tween 20+1% BSA+10% normal donkey serum for 1 h at room temperature. Primary antibodies were applied in 1xPBS+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, 1:1000), and histidine Decarboxylase (HDC) from rabbit (Progen, Cat. 16045, 1:500). Secondary antibodies were donkey IgGs coupled to Alexa dyes used at 1:500 for 2 h at room temperature.

## RESULTS

The Saudi Arabian family included 8 affected members (Figure 1) and was previously reported (BaHammam et al., 2008). At the time of investigations, 7 affected members were alive and all except one accepted to participate in the study (Table 1). Briefly, the 6 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, SD 1.5 years). Three were males and three were females. 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, while 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.

***Figure 1.***

***Table 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 a LOD score of 2.41 on chromosome 3 (Figure 2) between rs481319 (position Chr3:62,728,945) and rs7636827 (position Chr3:73,101,899). No other suggestive loci were found (LOD score >1.9 (Lander and Kruglyak, 1995), Figure 2). Haplotype analysis revealed a shared haplotype between all affected and one presumably unaffected (KLS1-17) subjects. The same analysis with a recessive model did not indicate any loci except a few single SNPs with LOD score > 1.9 (chrosome 1, 2, and 7).

***Figure 2.***

**Exome sequencing identified a missense variant in *LMOD3*:** Six members of the family including four affected and two unaffected (indicated by * in Figure 1), 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 candidate due to 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 3 regions with suggestive linkage on chromosome 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.*

**Resquencing 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 7 KLS patients (Table 2). The original p.E142D was found in a sporadic KLS case.

One variant (p.R83H) was found in 3 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, 3 low frequency (MAF<0.05) and two rare (MAF<0.01) variants were found in 7 independent KLS patients (Table 2). We have compared these variants to their frequencies reported in ExAC (Exome Aggregation Consortium) by Fisher 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 2 other sporadic KLS patients by the International Genetic Study Kleine-Levin Syndrome (University College of 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 2 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)

***Table 2.***

**LMOD3 is expressed in key brain structures:** Since 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 3A-D), dopamine (Figure 3I-H), serotonin (Figure 3M-P), noradrenaline (Figure 3Q-T), and most of histamine (Figure 3I-H) producing neurons, essential for the regulation of wakefulness, were LMOD3 positive.

***Figure 3.***

## 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 3 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 patient 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 due to the lack of a functional model (in a neuron-based system), although 3 out of the 5 variants are predicted by *in-silico* analysis to be pathogenic. Given that 7 out of our 38 cases (18.4 %) carried a *LMOD3* missense variant and 4 other sporadic cases (8.9%) from the UK exome sequencing project also carry 2 *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 were never been associated with another disease. KLS being a rare disease with an estimated prevalence of less than 5 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 unknown role in the nervous system. LMOD1 is extensively expressed in the brain (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 et al., 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 et al., 1982, Critchley, 1962, Gadoth et al., 2001, Malhotra et al., 1997, Takrani and Cronin, 1976). We found LMOD3 expression in the lateral hypothalamus, colocalized with hypocretin neurons. Potential implication of hypocretins (namely a decreased CSF level during the hypersomnia period) was reported (Lopez et al., 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, since KLS develops during adolescence with a favorable prognosis with age and our findings implicating LMOD3 variants, we propose that KLS might be a structural and/or developmental CNS disorder.

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

**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.

**Figure 3**: Expression of LMOD3 in the mouse brain. (A), (E), (I), (M), (Q) Coronal mouse brain sections at the level of Lateral Hypothalamus (LH), Tuberomammillary nucleus (TMN), Ventral Tegmental Area (VTA) and Substantia Nigra Pars Compacta (SNc), Dorsal Raphé (DR), and Locus Coeruleus (LC) at 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), (T). HCRT: hypocretin, LMOD3: leiomodin 3, HDC: Histidine Decarboxylase, TH: Tyrosine Hydroxylase, TPH: Tryptophan Hydroxylase. mt: mammillary tract, 3V: third ventricle, Aq: Aqueduct, 4V: Forth Ventricle. Blue color indicates 4',6-diamidino-2-phenylindole (DAPI) fluorescence of cell nuclei staining. Scale bars: 500 μm at low and 20 μm at high magnification.

**Table 1.** Demographic and clinical history of the Saudi Arabian family with KLS.

| Characteristics | | N; %; 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 time in episode, h/24 h | 12.7 ± 3.1 |
| Cognitive symptoms, % | Cognitive impairment | 100 |
| | Impaired speech | 100 |
| | Confusion | 100 |
| | Altered perception/derealization | 100 |
| | Apathy | 100 |
| Eating behavior disturbance, % | Megaphagia | 83.3 |
| | Decreased appetite | 16.7 |
| Sexual disturbances, % | Hypersexuality or disinhibition | 66.7 |
| Other psychiatric symptoms, % | Hallucinations/delusions | 16.7 |
| | Split body/mind, feeling of disembodiment | 100 |
| | Agitation/excitation | 66.7 |
| | Anxiety | 83.3 |
| | Depression | 83.3 |
| Meningeal symptoms, % | 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

**Table 2.** *LMOD* mutations found in sporadic KLS patients

| Patient | Position[a] | SNP | Mutation[b] | Amino Acid[c] | Inherited | MAF[d] |
|---|---|---|---|---|---|---|
| 1M | 69122139 | rs35740823 | c.G248A | p.R83H | Yes | 0.0439 |
| 2M | 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 |
| 5M | 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]Aminoacid, reference sequence NP_001291347.1

[d]MAF=minor allele frequency in non-Finnish Europeans from ExAC