

Multi-infarct dementia of Swedish type is caused by a 3'UTR mutation of *COL4A1*

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Running title: 3'UTR mutation of *COL4A1* causes hMID in a Swedish family

Cerebral small vessel diseases (cSVD) often present as sporadic conditions but several monogenic families have also been reported (Hagel *et al.*, 2004; Herve *et al.*, 2012). In 1977, Sourander and Wålinder described a family with an autosomal dominant cerebrovascular disease manifesting with transient ischaemic attacks/strokes, neuropsychiatric symptoms and progressive cognitive decline. Thirty years later it was proved that this family did not have mutations in *NOTCH3*, excluding the initially suspected diagnosis of cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL). Consequently it was concluded that this family presented a new cSVD, which was named hereditary multi-infarct dementia (hMID) of the Swedish type (Low *et al.*, 2007).

In order to identify the genetic cause of disease in this Swedish hMID family we performed whole-exome sequencing (WES) and genetic linkage analysis. Twenty-one family members participated in this study: 10 were affected, 10 were unaffected and one participant was an unrelated spouse used as a control (Figure 1A).

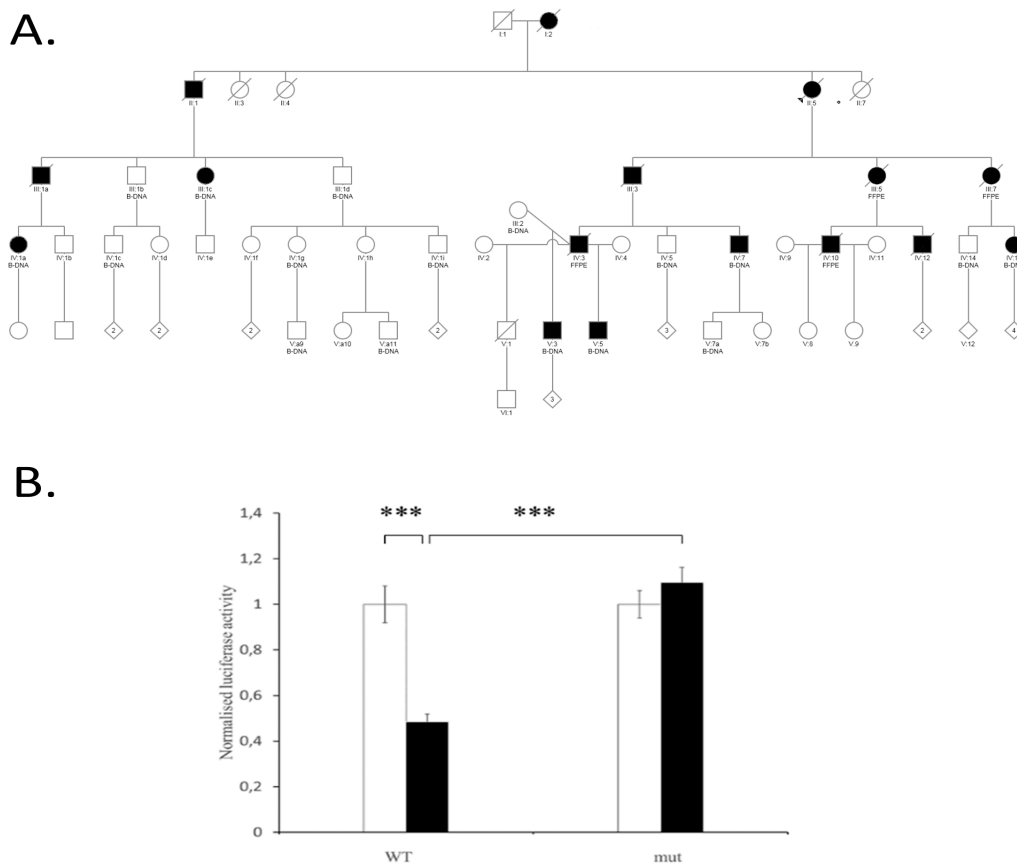


Figure 1: A. Pedigree of the Swedish hMID family. Family members included in the study are marked with B-DNA (blood derived DNA) or FFPE (formalin fixed paraffin embedded

tissue) according to the sample type available. Black symbols represent affected individuals and white symbols represent healthy family members. Diagonal lines indicate deceased individuals. The arrowhead indicates the proband (Individual II:5). B. Luciferase assay. HEK-293T cells were transfected with pMIR-REPORT luciferase wild-type (WT) or mutant (mut). Cells were co-transfected either with miR-29b-3p (black bars) or negative control microRNA (white bars). Normalized luciferase activity of cells transfected with the WT construct was significantly decreased by -miR-29b-3p, compared to cells transfected with a mimic-negative control. Luciferase activity was not altered in cells transfected with a mutated construct ($***p < 0.001$, 2-sided Student *t* test). Error bars indicate mean standard deviation.

Blood samples (n=17) were collected and DNA was extracted by standard methods after written informed consent was provided by all family members taking part in this study. For 4 patients only formalin fixed paraffin embedded (FFPE) tissues were available.

Four patients (III:1c, IV:7, IV:16 and V:3) and 2 unaffected family members (IV:5 and IV:14) were selected for WES. Exomes were prepared using the SeqCap EZ Human Exome Library version 2.0 (Roche Nimblegen Inc) and sequencing runs were performed on HiSeq 2000 (Illumina). Sequencing reads were aligned to GRCh37/hg19 using BWA (Li and Durbin, 2010) and variants were called according to GATK's standard best practices v3 (McKenna *et al.*, 2010; DePristo *et al.*, 2011). Following variant calling, annotation was performed using SnpEff (Cingolani *et al.*, 2012). For the linkage study, whole genome genotyping was performed for 12 blood-derived DNA samples using HumanOmniExpress Bead chips (Illumina). Parametric multipoint linkage analysis was performed using Allegro (Gudbjartsson *et al.*, 2000) under a fully penetrant autosomal dominant model.

Data analyses were based on an autosomal dominant mode of inheritance of the disease and the hypothesis that the underlying mutation was not present in neurologically healthy control individuals or in the general population (**Table 1**). Validation of variants found by WES was done using Sanger sequencing with BigDye Terminator version 3.1 chemistry (Applied Biosystems).

Parametric multipoint linkage analysis identified four peaks on chromosomes 10, 11, 12 and 13 achieving LOD scores > 2 . When these regions were compared to the WES data we identified three variants present in affected family members and absent in healthy relatives: one in *SPOCK2* and two variants in *COL4A1* (**Table 1**).

Table 1: Linkage regions with $LOD > 2$ and the variants identified by WES located in these chromosomal areas.

Chr	Chr location	dbSNP	LS	Variants	Gmaf
10	7349891-76372030	rs10823837- rs4746209	2,352	<i>SPOCK2</i> c.*11G>A	0,379
11	47929846- 49000550	rs6485795- rs11040198	2,294	No variants identified	
12	85165879- 87281210	rs11116595- rs7316774	2,348	No variants identified	
13	109327788- 111067000	rs9284246- rs10851243	2,407	<i>COL4A1</i> c.*32G>A <i>COL4A1</i> n.249C>T, c.4470C>T	- 0,376

Chr: Chromosome; *Chr location:* Chromosomal location (hg19); *dbSNP:* dbSNP accession numbers; *LS:* Logarithm of odds score; *Variants:* variants identified by WES in the region; *Gmaf:* global minor allele frequency in the Exome Aggregation Consortium (ExAC). Minor allele frequencies of 0,4 in the general population for both *SPOCK2:c.*11G>A* and *COL4A1:c.4470C>T* were considered to be too high for a mutation causative of a rare disease as Swedish hMID.

Only one of these variants (*COL4A1:c.*32G>A*) was found to segregate with the disease in the extended family and was absent from population databases. Although the variant is located in the 3'UTR of *COL4A1* both gnomAD and ExAC databases report variants in this locus in a minimum of 117,613 and 44,384 individuals, respectively (Lek *et al.*, 2016). The segregation of *COL4A1 c.*32G>A* with the disease was confirmed using Sanger sequencing. All affected cases had the variant and none of the older unaffected cases (age > 40 years) carried it. One younger, currently unaffected, family member also carried the *COL4A1 c.*32G>A*.

The *c.*32G>A* mutation is located in the 3' UTR region of *COL4A1*, and may affect the binding site of miR-29, located in this region. To test this hypothesis we performed a microRNA transfection study combined with luciferase reporter assay. 197 bp fragment of wild-type and mutated target site were amplified by PCR from patients' genomic DNA. The inserts were validated by sequencing. Amplified target region was digested with Hind III/Spe1, cloned into the pMIR REPORT Luciferase plasmid (AppliedBiosystems).

HEK293T cells (DMEM, 10% FCS serum with penicillin (100 U/ml) and streptomycin (100 µg/ml) in humidified air containing 5% CO₂ at 37°C) were plated in 24-well plates. At 80% confluence, 100 ng of empty, wild-type, or mutated plasmids were cotransfected with 25 pmol of either miRIDIAN hsa-miR-29b-3p or negative control (Dharmacon), using DharmaFECT Duo 2.5 µl in each well (Dharmacon). The triplicate samples were lysed with 1% NP40, 150 mM NaCl and 25 mM Tris, pH 7.6, and firefly luciferase activities were measured 36 hours after transfection using the 1000 Assay System (Promega) and analyzed with BioTek, Cytostation 5. The results suggested that the *COL4A1* c.*32G>A mutation affects miR-29 binding and hence leads to upregulation of *COL4A1* (**Figure 1B**).

COL4A1 mutations have been reported as the cause of a wide variety of autosomal dominant diseases being associated with variable phenotypes (Lemmens *et al.*, 2013). These include: porencephaly 1 (OMIM #175780); small vessel disease of the brain with or without ocular anomalies (BSVD, OMIM #607595); retinal arterial tortuosity (RATOR, OMIM #180000); hereditary angiopathy with nephropathy, aneurysms and muscle cramp (HANAC, OMIM #611773); Walker-Warburg syndrome (Labelle-Dumais *et al.*, 2011) and pontine autosomal dominant microangiopathy with leukoencephalopathy (PADMAL) (Verdura *et al.*, 2016).

Swedish hMID is a cerebral small vessel disease characterized by multifocal impaired cerebral blood flow resulting in multiple infarctions. Clinically and pathologically it fits within the expanding phenotypic group of *COL4A1* related disorders, most closely resembling PADMAL with lacunar infarcts in the subcortical and pontine areas (Sourander and Walinder, 1977; Hagel *et al.*, 2004; Verdura *et al.*, 2016).

The 3'UTR *COL4A1* variant identified here presented complete segregation with the disease in this family, being identified in all affected and absent in all older (>40 years of age) unaffected family members. One younger, healthy subject (V:7a) carried the *COL4A1* c.*32G>A variant, suggesting the possibility of currently being in an asymptomatic stage of the disease.

A recent publication by Verdura and colleagues identified mutations in *COL4A1* 3'UTR as the cause of cSVD in six families, including PADMAL cases. The mutations identified also affected the binding site for miR-29 micro-RNA located within the 3'UTR of *COL4A1*, and were shown to lead to upregulation of *COL4A1* mRNA expression (Verdura *et al.*, 2016). Although the variant found in this Swedish hMID family is novel, it disrupts the same miR-29 binding site, adding support to the pathogenicity of the mutation and suggesting that *COL4A1* upregulation is a central pathogenic mechanism both in Swedish hMID and PADMAL. The similarities at clinical and pathological levels also support this view: both

diseases are characterized by fibrohyalinosis and elastosis of small arterioles with atrophy of media and proliferation of the intima. These changes result in multiple lacunar infarcts in the basal ganglia, thalamus, periventricular white matter and pons, and in cortical and white matter atrophy. At the EM level, thickening of the basement membrane is observed in both diseases (Sourander and Walinder, 1977; Hagel *et al.*, 2004; Low *et al.*, 2007; Verdura *et al.*, 2016), and the clinical findings include cognitive impairment and progressive dementia, strokes, as well as mood and gait disturbances (Hagel *et al.*, 2004; Low *et al.*, 2007; Craggs *et al.*, 2013). In our previous study, immunostaining of COL4 revealed increased levels of staining in walls of small cerebral arteries both in PADMAL and Swedish hMID cases. However, the investigation of the sclerotic index showed some regional differences between the diseases: PADMAL seemed to affect the vessels of the frontal region more than those of the basal ganglia, whereas hMID cases showed the opposite effect (Craggs *et al.*, 2013). Furthermore, no haemorrhages have been described in subjects with PADMAL, while one hMID subject with anticoagulative treatment was reported to suffer from a massive haemorrhage (Sourander and Walinder, 1977).

As previously proposed, perturbations of the cerebrovascular matrisome (the group of proteins both constituting and associated with the extracellular matrix) can represent a convergent pathologic pathway in monogenic small vessel diseases (Joutel *et al.*, 2016). Still, further studies are needed to clarify the detailed pathogenic molecular mechanisms behind these diseases and to understand the phenotypic differences arising from mutations in the same micro-RNA binding site.

Legends for figures

Figure 1: A. Pedigree of the Swedish hMID family. Family members included in the study are marked with B-DNA (blood derived DNA) or FFPE (formalin fixed paraffin embedded tissue) according to the sample type available. Black symbols represent affected individuals and white symbols represent healthy family members. Diagonal lines indicate deceased individuals. The arrowhead indicates the proband (Individual II:5). B. Luciferase assay. HEK-293T cells were transfected with pMIR-REPORT luciferase wild-type (WT) or mutant (mut). Cells were co-transfected either with miR-29b-3p (black bars) or negative control microRNA (white bars). Normalized luciferase activity of cells transfected with the WT construct was significantly decreased by -miR-29b-3p, compared to cells transfected with a

mimic-negative control. Luciferase activity was not altered in cells transfected with a mutated construct (**p < 0.001, 2-sided Student t test). Error bars indicate mean standard deviation.

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Conflict of Interest and Sources of Funding

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