

Running Title Page

A novel 3'UTR mutation in *SLC29A3*

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Title Page

A novel 3'UTR mutation in the *SLC29A3* gene associated with pigmentary hypertrichosis and non-autoimmune insulin-dependent diabetes mellitus syndrome

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Location of Research

The work was carried out in London, UK

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Key words

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Abbreviations used

PHID: pigmentary hypertrichosis and non-autoimmune insulin-dependent diabetes mellitus

SLC29A3: solute carrier family 29 member 3

DM: diabetes mellitus

ENT3: equilibrative nucleoside transporter

3'UTR: 3' untranslated region

mRNA: messenger RNA

Introduction

Pigmentary hypertrichosis and non-autoimmune insulin-dependent diabetes mellitus (DM) syndrome, often referred to as PHID, is a rare autosomal recessive syndrome of severe multi-systemic inflammation that has only been described using the PHID terminology only a handful of times in the literature (1, 2). The PHID syndrome is an allelic variant of the H syndrome which is a cluster of disorders characterised by cutaneous hyperpigmentation, hearing impairment, heart abnormalities, hypertrichosis, hepatomegaly, hypogonadism and histiocytosis (3, 4). Additional features of the H syndrome can include short stature, hallux vagus, fixed flexion contractions of the proximal interphalangeal and toe joints in addition to lymphadenopathy (5, 6). The characteristic phenotype of this disease cluster is the cutaneous hyperpigmented, hypertrichotic and indurated patches that appear between the first and second decades of life (6). These pigmented plaques are histopathologically characterised by inflammation, excessive histiocytes, acanthosis in the basal layer of the skin and by the presence of excessive plasma cells in the dermis and subcutis (3, 7).

The overlapping features of the PHID and H syndromes include the hyperpigmented lesions and plaques particularly on the inner thighs, shins, genitals and abdomen, general hypertrichosis, perivascular lymphohistiocytosis and mild to moderate lymphadenopathy. Generally, but not always, prominent clinodactyly, sensorineural hearing loss and life threatening enlargement of the lymph nodes are features specific only to the H syndrome (5).

The frequency of DM in patients with PHID syndrome is about 83% and the DM is autoantibody negative. Typically, DM occurs in late childhood or early puberty and usually presents with diabetic ketoacidosis (4). Circulating insulin is absent in these patients and could not be induced by glucose administration attempts, confirming the abnormal production or secretion of insulin in PHID rather than insulin resistance (3). Moreover, severe exocrine pancreas insufficiency has been reported in two patients with PHID syndrome (2).

It is difficult to categorically separate the two syndromes as there have been reports of merging phenotypes between the two diseases (PHID and H syndromes) suggesting that these diseases should be grouped under one umbrella term. Table (1) elaborates on the published shared and differentiating features between the two syndromes that are continuously changing. However, the wide genetic heterogeneity of this disorder hinders the ability to draw strict and defining phenotypic categories and suggests that the two diseases might be part of one disease spectrum rather than two separate entities.

It has been established that the PHID and H syndromes are caused by protein changing mutations in the *SLC29A3* gene (10q22.1), demonstrating that these disorders belong to the spectrum of a single disease (4, 8). The *SLC29A3* gene encodes for the human equilibrative nucleoside transporter 3 (hENT3), which is part of a large conserved family of solute carrier transporters known as the ENT or SLC29 family (9). The ENT family members have a shared structure of 11 transmembrane alpha helices with an extracellular C terminus and a cytoplasmic N terminus (distinctive to ENT3) with a sizable cytoplasmic loop that joins transmembrane domains 6 and 7 (10). Transporters of nucleosides and nucleobases have a crucial cellular function since they play an integral role in nucleotide synthesis by mediating the uptake of nucleotide precursors by salvage pathways in various tissues (11) of organisms from different taxa including mammals, tunicates, teleost fish, insects, slime molds and round worms (12).

hENT3 is a sub-cellularly localised 475 amino acid protein that transports hydrophilic nucleosides, nucleobases and hydrophilic anticancer and antiviral nucleoside drugs (5). Unlike the other members of the ENT family that are membrane bound, ENT3 is partially localised to the late endosomes/ lysosomes where it acts as a pH dependent subcellular transporter. There

has also been reports that ENT3 is localised to the mitochondria where it acts as a mitochondrial transporter with an endosomal/ lysosomal targeting motif (11, 13).

Maintaining the nucleoside homeostasis is integral to the preservation of cellular integrity as they are essential for various cellular processes, especially the nucleoside salvage pathway. Also the nucleoside pool is crucial to the production of adenosine and guanosine triphosphates which are the foundation of cellular energy and signal transduction in the mitochondria (4, 11).

In an attempt to understand the role of ENT3 in the insulin signalling pathway it was demonstrated that the knockdown of the *Drosophilla SLC29A3* ortholog (*dENT1*) is semi – viable to lethal depending on the amount of ubiquitous loss. The knockouts died at different developmental stages revealing that *SLC29A3* is crucial for maintaining metabolic functions at different phases of development rather than being crucial for a single specific process. It was concluded that the expression of insulin receptors (*dPI3K* and *dAKT*) was able to rescue the abnormal phenotype secondary to the *dENT1* knockout, supporting the theory that *SLC29A3* is associated with the insulin signalling pathway components (4). This important study linked dysregulated *SLC29A3* expression to the PHID syndrome, confirming that this disease belongs to the H syndrome or *SLC29A3* spectrum disorders.

To explore the findings further, a reduction in mRNA and protein levels in the fibroblasts of a patient with a T449R mutation in *SLC29A3* was showed (4). Shortly after, studies have attempted to understand the effect of three other closely positioned mutations in the last cytoplasmic domain of ENT3 (14). They predicted that these mutations would cause a turnover increase after they reported that the degradation of ENT3 is mainly done through the lysosomal rather than the proteosomal pathway. Where turnover was slightly increased, it was concluded

(14) that the reduction in mRNA levels rather than protein stability is responsible for the reduction in transport function. Other mutants resulted in an accelerated turnover compared to the wild types, so it was concluded that decreased protein stability is also a contributor to the development of the *SLC29A3* diseases.

Consistently, it was shown (4) by fluorescent microscopy that the combination of decreased protein levels, the likely impaired protein functionality and the accumulation of ENT3 in the late endosomes/lysosomes are responsible for the disease development.

In this paper we describe a novel *SLC29A3* variant in the 3'UTR, a genomic region which remain highly under explored. The aim of our study is to assess the pathogenicity of this mutation which could shed a light on a potentially new genetic mechanism of this syndrome.

Materials

Subjects

Two male siblings aged 20 (patient M) and 15 years (patient N) respectively, both born from a consanguineous marriage in Turkey, presented with typical manifestations of the PHID syndrome at the outpatient clinic of Paediatric Endocrinology in the Istanbul Faculty of Medicine, Istanbul University. Figure 1 shows the clinical features of the two siblings along with the family pedigree.

Patients M and Patient N initially presented with a complaint of severe growth failure, at the age of 11 and 6 years, respectively. Their paternal uncle had growth failure, persistent diarrhea and pigmentary hypertrichosis. He died at the age of 2.5 years due severe diarrhea and samples could not be obtained for the purposes of this study. The uncle did not have DM. Both parents have normal development.

Patient M was born at 39 weeks of gestation via vaginal delivery following an uncomplicated pregnancy. He was small for gestational age, weighing 2000 grams (-3.8 SDS). Neuromotor development was normal. This patient was brought to our care at the age of 4 years with complaints of diarrhea, hyperpigmentation and hypertrichosis on his back, arms and legs. He was diagnosed with chronic diarrhea due to exocrine pancreas insufficiency after having prolonged diarrhea (>4 weeks) with evidence of fat and protein malabsorption in stool analysis that also showed reduced chymotrypsin activity (4.5 U/gm, normal range: > 6) .Pancreatic enzyme replacement therapy was started with good response. At the age of 9 years, he was diagnosed with type 1 DM following the manifestations of polyuria and polydipsia after which he was started on insulin therapy. Islet cell, insulin and glutamic acid decarboxylase antibodies were negative. On subsequent evaluation at the age of 11 years, his height was 113 cm (-4.8 SDS), weight was 23.4 kg (-2.7 SDS), BMI was 18.3 kg/m² (0.02 SDS) and sitting height/height ratio was 0.54 (normal range: 0.5-0.55). At this point his puberty had just started

and pubertal stage was Tanner 2 (Ax1 Ph2 testes: 6/6ml) at presentation. He had hyperpigmented hypertrichosis on his back, arms and legs. There was evidence of hepatomegaly (4cm) and no splenomegaly and lymphadenopathy was identified. He also had camptodactyly and mild hallux valgus. Laboratory investigations showed a blood glucose level of 684 mg/dl - 38 mmol/L (normal range: 70-100 mg/dl , 3.9-5.5 mmol/L), HbA1C was 12.8 % (normal range: 4.8-6.0%), ALT (122 U/L, normal range: 13-45 U/L), AST (91 U/L, normal range: 5-40 U/L), and triglyceride levels (561 mg/dl, normal range: <170 mg/dl) were high. Pancreatic enzymes, serum amylase (21U/L, normal range: 25-110 U/L) and lipase (10 U/L, normal range: 10-60 U/L) were mildly low. Free thyroxine (FT4) was mildly low (12pmol/L, normal range: 12 -22 pmol/L), TSH was normal, cortisol and prolactin levels were normal. IGF1 (<25 ng/ml, normal ranges: 75-420 ng/ml) and IGFBP-3 (1240 ng/ml, normal range: 2300-6300 ng/ml) were low. Bone age (9 years) was delayed. L-thyroxine treatment was started because of secondary hypothyroidism. Insulin and pancreatic enzyme replacement treatment were continued. Stimulation tests of growth hormones revealed partial growth hormone deficiency (growth hormone peak response in the clonidine and L-dopa tests were 8.0 ng/ml and 6.9 ng/ml, respectively, with the normal response range being ≥ 10 ng/ml). Growth hormone treatment was started at the age of 12.5 years when growth velocity was slow. This treatment was continued until the age of 17.5 years. At the most recent physical examination at the age of 19 years, his height was 152.2 cm (-3.8 SDS), weight was 51.8 kg (-2.4 SDS), head circumference was 52.5 cm (-3.4 SDS) and BMI was 22.4 kg/m² (-0.2 SDS). HbA1C was 8.5 % (normal range: 4.8-6.0%). Cardiologic and ophthalmologic examinations, electromyography, echocardiography and audiometry, cranial and pituitary magnetic resonance imaging (MRI) were all normal. His pubertal progression and gonadal hormones levels were within normal ranges.

At the most recent physical examination at the age of 19 years, height was 152.2 cm (-3.8 SDS), weight was 51.8 kg (-2.4 SDS), head circumference was 52.5 cm (-3.4 SDS) and BMI was 22.4 kg/m² (-0.2 SDS). Pubertal stage was Tanner stage 5. HbA1C was 8.5 % (normal range: 4.8-6.0%). The patient had non-autoimmune type 1 DM, secondary hypothyroidism, hypertrichosis and hyperpigmentation. Insulin, pancreatic enzyme replacement therapy and L-thyroxine replacement therapy were still being used.

Patient N was born at 38 weeks of gestation with a birth weight of 3250 grams (0 SDS) by caesarian delivery following an uncomplicated pregnancy. There was no perinatal asphyxia. Neuromotor development was normal. This patient was brought to our care at age of 4 years, secondary to polyuria, polydipsia and diarrhoea. Diarrhoea was persistent for 2 years. Similarly to his brother (patient M), he was diagnosed with chronic diarrhoea secondary to exocrine pancreas insufficiency and type 1 DM. Pancreatic enzyme replacement and insulin treatment were started. On subsequent examination at the age of 6 years, his height was 94.3 cm (-5.5 SDS), weight was 16.9 kg (-2.3 SDS), BMI was 19 kg/m² (1.7 SDS) and sitting height/height ratio was 0.54 (normal range: 0.5-0.55). He had hepatomegaly (6 cm) and abdominal distention. There was no evidence of splenomegaly and lymphadenopathy. His skin and skeletal findings were similar to Patient M. Blood glucose was 143 mg/dl - 7.9 mmol/l (normal range: 70-100 mg/dl, 3.9-5.5 mmol/L), HbA1C was 9.2 % (normal range: 4.8-6.0%). ALT (80 U/L, normal range: 13-45 U/L) and AST (46 U/L, normal range: 5-40 U/L) were mildly high. FT4 was low (5.8 pmol/L, normal range: 12-22 pmol/L) and TSH (5.81 mIU/L, normal range: 0.6-10 mIU/L), cortisol and prolactin levels were within normal ranges. Stool analysis revealed malabsorption of fat and protein diagnosed by the presence of overt steatorrhea and reduced chymotrypsin activity (2.5 U/gm, normal range: > 6 U/gm). Serum IGF-1 (<25 ng/ml, normal range: 52-297 ng/ml) was low and IGFBP-3 (2960 ng/ml, normal range: 1200-5600 ng/ml)

levels were low. Stimulation testing for growth hormone deficiency was performed and the results showed a growth hormone peak response of 1.98 ng/ml and 2.21 ng/ml in the clonidine and L-dopa tests, respectively (normal response: ≥ 10 ng/ml). Growth hormone treatment was started at the age of 7.5 years. Abdominal ultrasonography revealed that the liver was enlarged with pancreatic hypoplasia. Liver biopsy showed mildly mononuclear cells infiltration in the portal areas. Other clinical and laboratory findings of this patient were similar to his brother. This patient also had non-autoimmune type 1 DM, exocrine pancreas insufficiency, secondary hypothyroidism, pigmented hypertrichosis and growth hormone deficiency. Insulin and pancreatic exocrine enzymes replacement treatment were continued and L-thyroxine was also started.

At the last physical examination at the age of 13.8 years, the patient's height was 128 cm (-4.6 SDS), weight was 32 kg (-2.8 SDS), head circumference was 51.1 cm (-3.1 SDS) and BMI was 19.5 kg/m^2 (-0.3 SDS). HbA1C was 6.6 % (normal range: 4.8-6.0%). Pubertal stage was Tanner 2 (Ax1Ph2 Testes 5/5 ml) with LH, FSH and testosterone levels in the normal ranges. HbA1C was 6.6 % and bone age was 7.5 years. Insulin, pancreatic exocrine enzymes, growth hormone and L-thyroxine replacement therapy were still continued. Cardiologic and ophthalmologic examinations, echocardiography, electromyography, audiometry, and cranial/pituitary MRI screenings were normal, similarly to patient M.

Methods

Consent was obtained from the patients and their caregivers (where applicable) prior to their inclusion in this study. Ethical consent for this study was granted by the Research & Development office at UCL GOS Institute of Child Health. Handling of patient samples was in accordance with the declaration of Helsinki. A blood sample and a 4mm skin punch biopsy was collected from each patient in the Istanbul faculty of medicine hospital and was sent to UCL GOS Institute of Child Health for genetic and molecular analysis.

Genomic DNA was extracted from the patients' bloods at GOSH North East Thames Regional Genetics Service Laboratories using the Maxwell 16 Blood DNA Purification Kit (Promega, USA).

SLC29A3 primers (supplementary table 1) were designed using the Primer3 software and obtained from Sigma-Aldrich (USA) in a lyophilized state. DNA was amplified and Sanger sequenced using standard methods (PCR cycling conditions available in supplementary table 1). Primary fibroblast cell lines were established for each patient in a biosafety level-2 cell culture laboratory and were cultured using Dulbecco's Modified Eagle Medium GlutaMAX supplement (Thermo Fisher Scientific, USA) and Fetal Bovine Serum from South American origins (Thermo Fisher Scientific, USA).

Protein expression of the encoded protein ENT3 was analysed by western blotting using an *SLC29A3* anti-rabbit antibody (Thermo Fisher Scientific, USA, #PA5-38039) and a *GAPDH* anti-rabbit housekeeping antibody (Cell Signalling Technology, USA, #2118S).

For mRNA expression analysis, complementary DNA (cDNA) was synthesized in a reverse transcription PCR reaction using RNA isolated from the patients' fibroblasts using the RNA easy kit (Qiagen, USA, #74104). Real time quantitative PCR was performed by using *Power SYBR Green* PCR master mix (PCR cycling conditions available in supplementary table 1). *SLC29A3* and endogenous control *RPL19* primers were designed and used in the real-time

quantitative PCR experiment that compared their relative amplification using the comparative CT method.

Whole exome sequencing was done at UCL Institute of Neurology using the Illumina HiSeq 2000 platform (Illumina, San Diego, USA). The sample enrichment and library preparation were based on the Agilent SureSelect v4 protocols (Agilent, Santa Clara, USA). Samples were sequenced at a final coverage of 30x. Data interpretation was done using the Ingenuity Variant Analysis (Qiagen, USA) software.

Results

SLC29A3 was Sanger sequenced in a candidate gene approach as the patients' phenotype was consistent with the clinical symptoms of the PHID syndrome described in the literature. A novel 3'UTR homozygous missense variant, c.1893G>A, was identified in patients M and N. The parents were found to be both heterozygous (c.1893G>A) and their sister has the wild type allele (G) only (Figure 2).

As the variant was found in the non-protein coding 3'UTR of *SLC29A3*, the pathogenicity of this change required further investigations. The patients' skin biopsies alongside seven control cell lines obtained from the Camelia Botnar laboratories at GOSH and from the department of Genetics and Genomic Medicine at UCL GOS ICH were cultured using standard cell culture techniques and were controlled for passage number, RNA and protein extraction methods. The control cell lines were treated identically to the two patient cell lines. RNA expression was analysed by the quantitative real time PCR method which revealed decreased expression levels by 47% in the patients compared to the controls in an average of 4 controlled runs (figure 3).

To assess the pathogenicity of this variant further, protein expression analysis of hENT3 was performed by lysing the patient and control cell pellets under identical conditions. Western blotting results showed a clearly visible decrease in the ENT3 protein levels in the patient cells compared to the controls (Figure 4).

In order to rule out any possibility of having missed any mutation in the coding regions of *SLC29A3* that could explain our findings, whole exome sequencing was performed. The results confirmed our Sanger sequencing findings that there are no mutations in any of the protein

coding exons of *SLC29A3* which strengthens our confidence in the pathogenicity of the c.1893G>A 3'UTR variant discovered.

Discussion

A candidate gene approach was initially undertaken since the hyperpigmentation and hypertrichosis accompanied by DM are the distinctive features of the PHID syndrome. No mutations were found in any of the 6 protein coding exons of *SLC29A3*. Instead, a novel 3'UTR variant, *SLC29A3* c.1893G>A was identified in the two siblings. Subsequently, two primary cell lines were established from these patients' skin biopsies and functional experiments were planned to investigate whether this change is indeed causing the patients' distinctive phenotype.

SLC29A3 expression was assessed by comparing the RNA levels of the patients to seven controls that were matched for passage numbers and RNA extraction methods. Two primer sets were used for each run that was repeated four times, after which an average expression was obtained. In every run, the expression levels in the patients' fibroblasts were consistently lower than the controls (figure 3A). The average *SLC29A3* expression in the patients is decreased by almost half compared to the controls (figure 3B).

Since only two patients were available, statistical comparisons could not be done between the two groups (controls vs patients). From the RT-PCR data, it can be concluded that the c.1893G>A mutation does lead to a decrease in *SLC29A3* expression by altering the stability of the hENT3 mRNA. This is consistent with literature findings (4) which has showed that the hENT3 mRNA levels in fibroblasts with a protein changing mutation (T449R) in *SLC29A3* were reduced to 34% compared to eight control cell lines. Additionally, the hENT3 levels were examined by western blotting which showed a clear decrease in the patients' hENT3 levels compared to the controls.

This cumulative functional data supports the notion that the *SLC29A3* c.1893G>A 3'UTR variant identified in the patients is likely to be disease causing as it affects the mRNA stability and expression levels of *SLC29A3*, which in turn leads to a decrease in hENT3 protein levels.

Since a candidate gene approach to sequencing can be biased, whole exome sequencing was performed as well. This was to check for any missed mutations in the coding regions of *SLC29A3* and to check for any other potential candidate gene (s). This endeavour did not generate any additional information as no protein changing mutations in *SLC29A3* were identified and no other suitable candidate genes were found.

There is a growing body of literature on the role of the 3' and 5' UTRs in disease development such as in myotonic dystrophy (15), amyotrophic lateral sclerosis (16) and chronic heart disease (17). It has been established that translational regulation of gene expression is equally crucial to the cellular functions as transcriptional regulation and the disruption of either of these processes can result in pathology (18, 19). Translational regulation and ability is based on the prolonged interaction between the different structures and components of the 3' and 5' UTRs. These factors include the 5'- cap, upstream open reading frames, secondary structures, various upstream AUGs, internal ribosome entry sites (IRESs) and polyadenylation signals such as the iron-responsive elements (IREs) which create networks with act trans- acting components (19).

Transcriptional regulation is mediated by an interplay of transcription factors, an RNA polymerase and a group of cis acting DNA components such as enhancers, promoters, silencers and locus control elements. These structures are arranged in a modular pattern where they regulate the generation of pre-mRNA which then go through a cascade of processing events to become mature mRNA. Initially the introns are removed, then a m7G (7-methyl-guanylate)

cap is placed at the 5' end of the first exon followed by the addition of the poly(A) tail which consists of 100-250 adenine residues at the 3' end of the rear exon which is a product of the primary transcript cleavage (15).

One of the main roles of the UTRs is post transcriptional regulation of gene expression which is done by several processes. These include ensuring the efficient transport of mRNAs out of the nucleus and modulating their subsequent subcellular localization and stability (20, 21). The crucial role of the UTRs in gene expression regulation is highlighted by the fact that mutations in this region have been linked to various pathologies (15, 16, and 17).

The fact that the 3'UTR is not limited by any structural constraints (ie: less introns) like the 5'UTR makes it a hotspot for pathologies (22). Variations in the 3'UTR can lead to pathologies by affecting the expression of the one gene in which the 3'UTR mutation is residing or by affecting the expression of several genes. The latter can be achieved by inflicting changes in one or more trans-acting factors affecting the fate of multiple mRNA molecules. Consequently, the 3'UTR transcribed from the mRNA molecule affected by the mutation can exert a dominant negative effect by hindering the trans-acting regulatory proteins and/ or transport (15, 22).

This evidence regarding the importance of the 3'UTR in disease development strengthens our findings and encourages endeavours of searching for genetic pathologies in the whole genome rather than the protein coding regions only.

Conclusion and limitations

This study provides the first report that links a 3'UTR variant to the PHID syndrome specifically and to the rare syndromes of DM in general. This finding highlights the importance of checking for variants in the 3'UTR that are most often ignored. In the majority of the cases, only the coding genomic regions are screened for the known genetic diseases which can be easily missed. If the UTRs were routinely screened and understood, the number of diagnoses of certain rare diseases might increase. For instance, the PHID syndrome has only been reported a handful of times in the literature and that may be due to the combination of this syndrome's rarity and to the missed genetic diagnoses. Screening the *SLC29A3* 3'UTR might possibly increase the numbers of PHID diagnoses.

Limitations of this study are mainly due to the limited patient numbers which is expected in the study of rare diseases. The small sample number hindered our ability to perform any statistical analyses when comparing the RNA and protein expression levels of the patients and the controls. Also, the inability of obtaining samples that could have strengthened causality from our patients' paternal uncle with a similar phenotype was another drawback.

In summary, this paper provides evidence to support the pathogenicity of a novel 3'UTR mutation identified in the *SLC29A3* gene in two Turkish patients with a PHID phenotype. This is the first report of a 3'UTR mutation in the *SLC29A3* spectrum disorders which opens the possibility of checking the 3'UTR before ruling out this diagnosis. The pathogenicity of this mutation was assessed by mRNA and protein expression levels which both showed altered expression levels in the mutant cell lines, which is consistent with the findings of protein changing mutations in *SLC29A3* (4).

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Legends

Table 1. Overlapping and differentiating clinical features of the H and PHID syndromes based on published case reports.

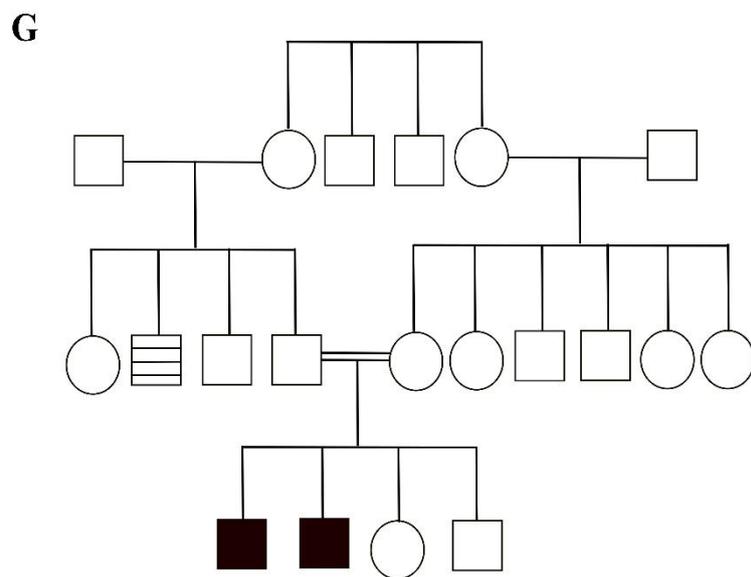
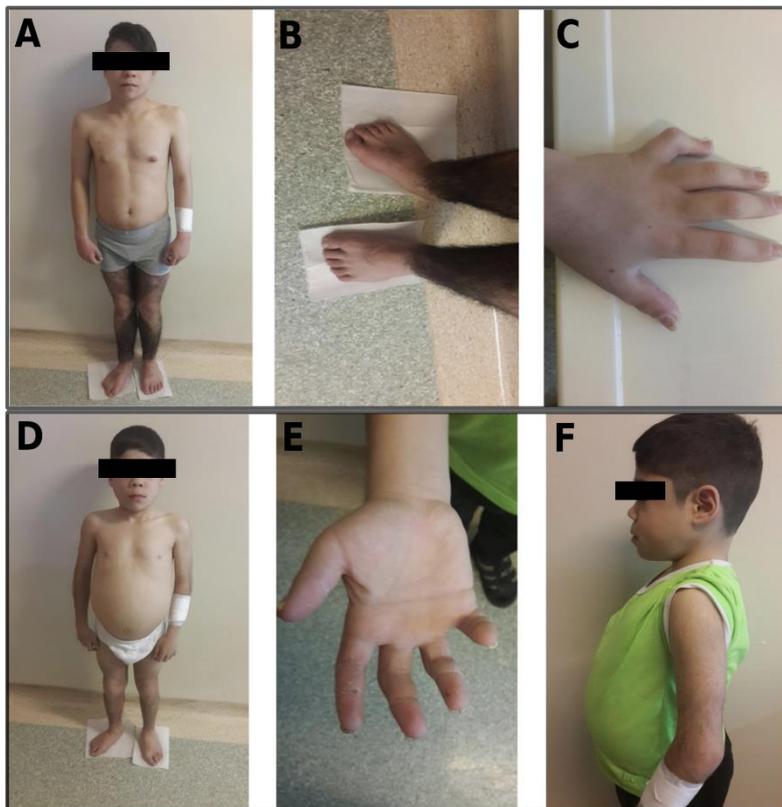
Figure 1. Clinical features and pedigree of patients M and N. (A → C: patient M), (D → F: patient N). (A & D) Hyperpigmentation and hypertrichosis in the legs. (B) Pronounced hypertrichosis with mild hallux valgus. (C & E) Camptodactyly. (F) Hypertrichosis on the hands. (G) Family pedigree of patients M and N.

Figure 2. Genetic studies showing a novel mutation in the 3'UTR of *SLC29A3*. DNA Sanger sequencing chromatogram showing a homozygous mutation *SLC29A3* c.1893G>A in patients M and N. The patients' parents are both heterozygous *SLC29A3* c.1893G>A and the patients' sister has the wild-type allele *SLC29A3* c.1893G.

Figure 3. mRNA expression studies showing a decrease in *SLC29A3* expression in patients M and N compared to the controls. (A) The *SLC29A3* expression levels are consistently lower in patients M and N compared to the seven control samples, measured over four runs of qRT-PCR. (B) On average the *SLC29A3* expression in patients M and N is reduced by 47%.

Figure 4. Protein expression studies showing a decrease in ENT3 levels in patients M and N compared to two controls. (A) Western blot showing almost undetectable levels of ENT3 in both patients M and N compared to the controls. (B & C) Densitometry results showing a reduction of ENT3 expression by 85.7% in the patients compared to the controls.

Figure 1



■ Patients M & N
▨ Paternal uncle with similar phenotype to patients M & N

Figure 2

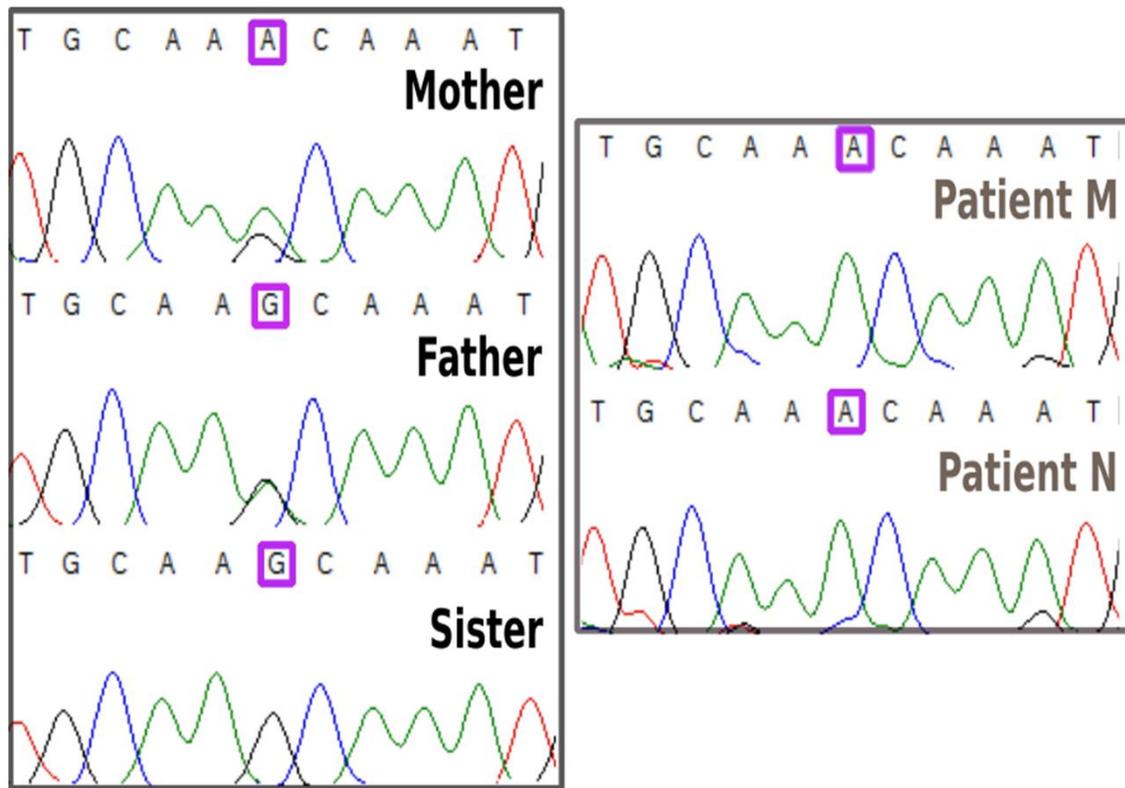


Figure 3

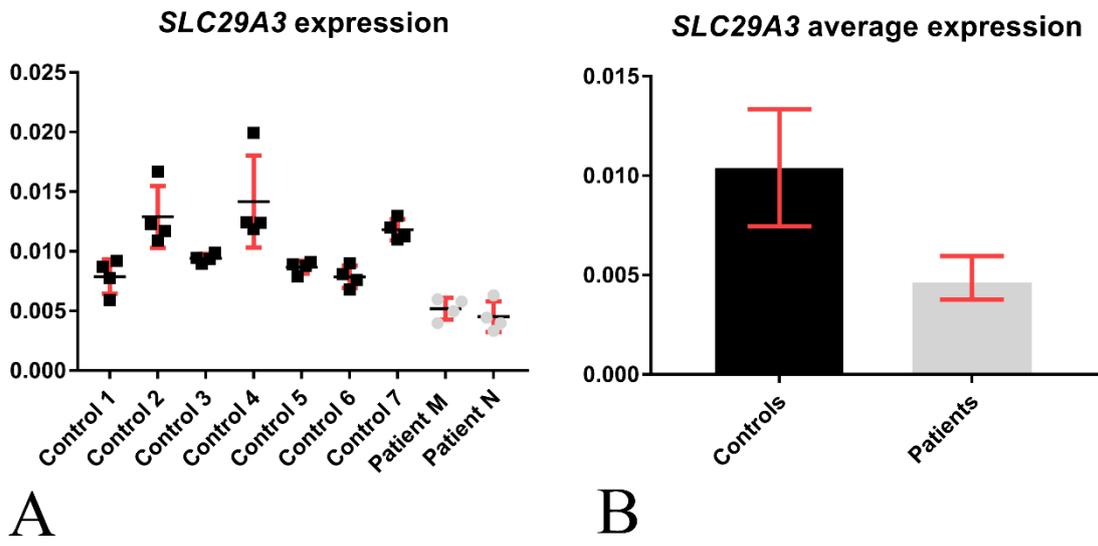


Figure 4

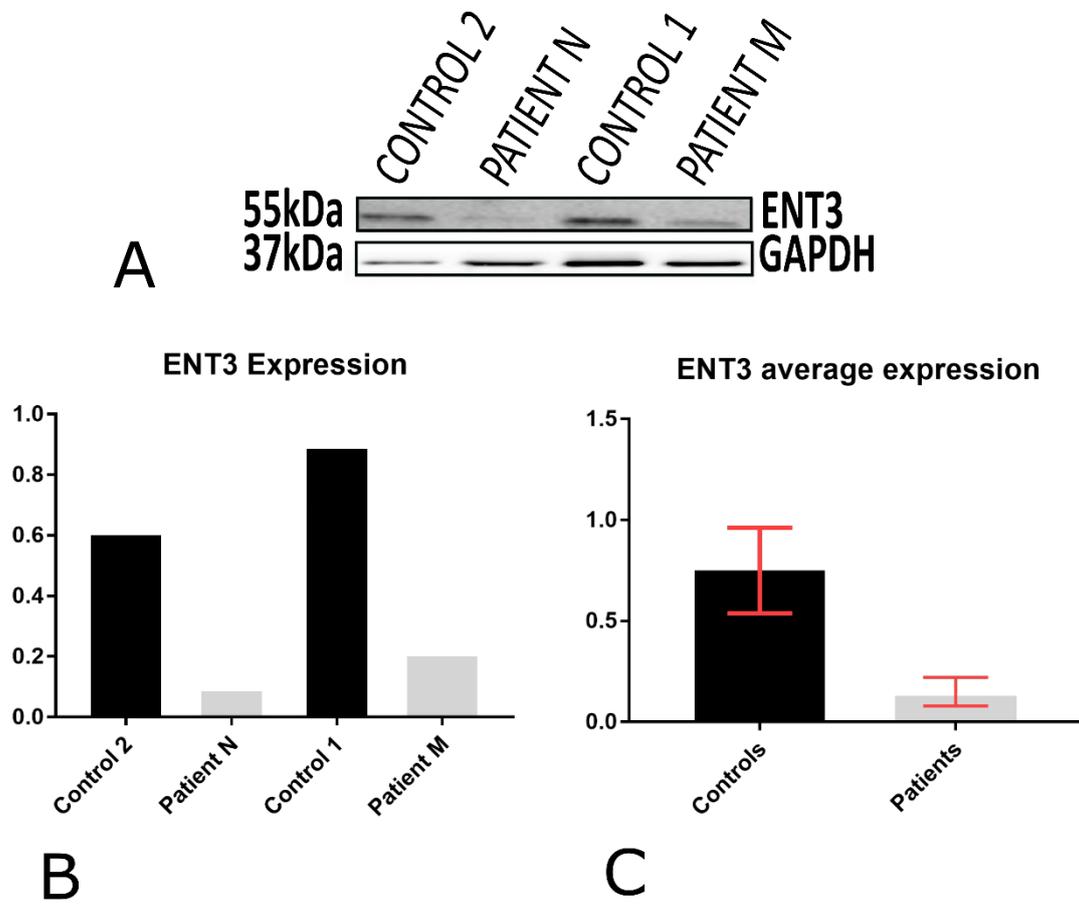


Table 1

Clinical features	H syndrome	PHID syndrome
Skin	Hyperpigmented and hypertrichotic skin lesions (9)	
Heart	Atrial septic defects, pulmonary stenosis, patent ductus arteriosus (1)	Cardiomyopathy (7)
Ear	Sensorineural deafness (5)	No deafness (2)
Abdomen	Hepatosplenomegaly (9)	
Pancreas	DM in some cases (23%) (9)	DM in the majority of cases (>80%) & severe pancreatic exocrine deficiency (2)
Eyes	Exophthalmus with normal thyroid function (9)	-
Growth	Short stature (2)	
Endocrine	Hypogonadism (3)	Delayed puberty (2)
Hands	Camptodactyly and flexion contractures (5)	-
Feet	Hallux valgus and fixed flexion contractures of toe joints (9)	-
Haematological features	Histiocytosis (9)	
Lymph nodes	Lymphadenopathy (9)	

Method	PCR forward primer	PCR reverse primer	PCR conditions
DNA sequencing	CCGGCAGAAGACAAGG AA	GGTTCCTGACTCTGGCTAG G	Standard PCR 35 cycles: 95°C for 5 minutes, 95°C for 1 minute, 60°C for 30 seconds, 72°C for 1 minute.
	GACTTTACAGAGCCCAG GGT	TCTCCATCTTCCCCTGCAT G	
	TGAAGACAGTGGGGAGA AGC	CCACCACTTAAGTAGGCA GAAA	
	GCTCGCCTGCTTCCCTTA A	ACATGCTCATCTCTGGCTC C	
	CTCCCTGTCTCTGAGGCT TC	CCACCAGCCAAGCCTATT TG	
	CTGGGCTGGAAGGTTCT GTT	GCACAATCTTAGGCCCGT AG	
	CCGCGTCCACCTGAAGA C	GCTCACTGGCATCTTGGG	
	GAAGGGAGGACACAAG GACA	TGCCTAATCTGAGTGTGCC A	
Allele specific quantitative real	TGGGCCTTCCATGAATG CTT	AGACCACCAGGTCATGAG GA	Standard protocol as per manufacturer's

<p>time PCR assay</p>			<p>guidelines for SYBR® Green real time PCR using the StepOne plus instrument.</p>
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Supplementary table 1: Primer sequences and PCR conditions applied for the amplification of *SLC29A3*.