BRIEF REPORT

Severe Infantile Isolated Exocrine Pancreatic Insufficiency Caused by the Complete Functional Loss of the *SPINK1* Gene

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Abstract

Exocrine pancreatic insufficiency (EPI) is rare in children, with most if not all cases occurring as part of syndromic conditions such as cystic fibrosis and Shwachman-Diamond syndrome. Here we report two cases, both presenting with severe EPI around five months of age. Characterized by diffuse pancreatic lipomatosis, they otherwise exhibited no remarkable deficiencies in other organs. Novel non-identical homozygous variants (a deletion removing the entire *SPINK1* gene and an insertion of a full-length inverted *Alu* element into the 3'-untranslated region of the *SPINK1* gene) resulting in the complete functional loss of the *SPINK1* gene (encoding pancreatic secretory trypsin inhibitor) were identified in each patient. Having correlated our findings with current knowledge of SPINK1's role in exocrine pancreas pathophysiology, we propose that complete and partial functional losses of the *SPINK1* gene are associated with quite distinct phenotypes, the former causing a new pediatric disease entity of severe infantile isolated EPI.

KEY WORDS: *Alu* insertion; chronic pancreatitis; exocrine pancreatic insufficiency; pancreatic lipomatosis; *SPINK1* gene

Exocrine pancreatic insufficiency (EPI) is a condition characterized by deficiency of the exocrine pancreatic enzymes, resulting in an inability to digest food properly. The hallmark symptoms of severe EPI are weight loss and loose fatty stools termed steatorrhea (Struyvenberg et al., 2017). Chronic pancreatitis is the leading cause of EPI in adults (Pezzilli et al., 2013). Severe EPI due to chronic pancreatitis in children is rare for two interrelated reasons. First, chronic pancreatitis is a persistent inflammatory process of the pancreas, generally requiring a significant period of time for EPI to develop (Layer et al., 1994). Second, severe EPI appears only once pancreatic enzyme output falls below 5-10% of normal (DiMagno et al., 1973). Characteristic radiographic features of chronic pancreatitis include pancreatic ductal dilation and parenchymal calcification (Majumder and Chari, 2016).

The primary causes of severe EPI in children are cystic fibrosis and Shwachman-Diamond syndrome (Buscail and Carrière, 2010), which are caused by mutations in the *CFTR* (MIM# 602421) and *SBDS* (MIM# 607444) genes (Boocock et al., 2003; Kerem et al., 1989), respectively. Besides EPI, affected patients typically display other abnormalities e.g. lung inflammation/infection in cystic fibrosis, and hematological abnormalities with single- or multi-lineage cytopenias and bone abnormalities in Shwachman-Diamond syndrome. The typical pathological finding in the pancreas of patients with Shwachman-Diamond syndrome is fatty infiltration, termed pancreatic lipomatosis (Robberecht et al., 1985). Pancreatic lipomatosis is also a frequent finding in older patients with cystic fibrosis (Soyer et al., 1999). Severe EPI in children can also arise from some other

rarer syndromic conditions such as Pearson syndrome and Johanson-Blizzard syndrome. Nonetheless, in all the aforementioned syndromic conditions, a clinical diagnosis can usually be easily made, due to their corresponding hallmark phenotypic manifestations. Herein we describe the first report of two cases of severe infantile isolated EPI and the identification of their underlying genetic causes.

Case 1 was born at term, with a normal weight (3310 g), height (47 cm) and head circumference (33 cm), to first cousin consanguineous parents of Turkish origin (Fig. 1A). The patient's family medical history was unremarkable. At five months of age, she presented with failure to thrive (weight, 4.940 kg, −2.5 SD; height, 54 cm, −3 SD; head circumference, 38 cm, −3 SD) and erythemato-squamous skin lesions. She was exclusively breast-fed and did not have bowel problems. Clinical examination revealed facial dysmorphism (large cheeks, nasal alae hypoplasia, crooked and brittle hair), but no other anomaly – no hepatosplenomegaly in particular. Clinical laboratory tests showed undernutrition, malabsorption, and mixed cytolytic and cholestatic liver damage without liver cell insufficiency (Supp. Table S1). The patient's metabolic (ammonemia, blood and urine carnitine level, blood acylcarnitine analysis, blood amino acid and urine organic acid chromatographies) and immune (lymphocyte subpopulation counts, serum immunoglobulin concentrations, vaccine-induced antibody titers) function was found to be normal. In addition, no immunoallergic reactions were discovered: radioallergosorbent tests for cow's milk protein, anti-trans-glutaminase antibodies and anti-enterocyte antibodies were all negative. Skin biopsies revealed psoriasis-like dermatitis (parakeratosis without necrosis). Hair analysis showed the absence of hair breaks or nodosities, allowing us to rule out tricho-hepato-enteric syndrome, and pointing instead towards nutritional deficiency. The patient's gastroscopy and colonoscopy were both normal; however, an abdominal ultrasound scan revealed a hyperechogenic pancreas of normal size. Magnetic resonance imaging of the

patient's pancreas performed secondarily at five years of age revealed major fatty infiltration throughout (Fig. 1B; Supp. Fig. S1). The patient's fecal elastase concentration (5 μg/g, N > 200) and steatorrhea after fat intake (7.8 g/d) confirmed the diagnosis of EPI. Pancreatic enzyme replacement therapy was initiated, and the patient was given parenteral nutrition for two weeks with a gradual reintroduction of enteral feeding using a hypoallergenic infant formula (Peptijunior, Picot) containing extensively hydrolyzed proteins. Subsequently, her liver function and growth rate returned to normal and her skin lesions disappeared. At six years of age, her weight, height and head circumference were 18.2 kg, 105.5 cm and 51 cm, respectively and she was symptom-free with normal intellectual development.

Case 2 was born at term, with a normal weight (2700 g) and height (50 cm), to first cousin consanguineous parents of Algerian origin (Fig. 2A). She had fatty stools since five months of age. When she was first examined by us at 32 months of age, her weight was 14.3 kg (+1 SD) and her height was 93 cm (+1 SD); a 24-hour fecal fat test showed 45.5 g/d fat (normal, around 1), chymotrypsin activity of 0.4 Q/g (normal, > 6) and an elastase concentration of < 15 mg/g (normal, > 200) in the stool; serum concentrations of vitamins D, A and E were significantly decreased; all other laboratory and clinical examinations were normal. Magnetic resonance imaging performed at three years of age showed a normal sized pancreas with diffuse lipomatosis but with neither dilatation of the canal of Wirsung nor presence of intraparenchymal nodules; her liver, kidneys, spleen and gallbladder showed normal morphologies and were all of normal size. She received enzyme replacement therapy and, when was examined at five years of age, was 21 kg (+1.5 SD) in weight and 116 cm (+2 SD) in height. Her father and paternal grandmother had complained of recurrent abdominal pain.

Cases 1 and 2 were initially analyzed for mutations in the *CFTR* and *SBDS* genes, both yielding negative findings. They were then analyzed for mutations in three trypsin-dependent pathway genes causing chronic pancreatitis (Hegyi and Sahin-Tóth, 2017), namely *PRSS1* (MIM# 276000), *SPINK1* (MIM# 167790) and *CTRC* (MIM#

601405) (Le Maréchal et al., 2006; Masson et al., 2008; Rosendahl et al., 2008; Whitcomb et al., 1996; Witt et al., 2000). No disease-causing mutations were found in the *PRSS1* and *CTRC* genes whilst two novel *SPINK1* gene mutations were found in the homozygous state. Methods pertaining to mutational analyses of the *SPINK1* gene as well as the functional analyses described below are provided in Supp. Methods as well as in Supp. Table S2 and Fig. S2. Written consent was obtained from each patient's parents and the Ethical Committees of the involved services approved genetic analyses.

In case 1, high-resolution DNA melting (HRM) analysis of the patient indicated no amplification of any of the four exons of the *SPINK1* gene, suggesting a homozygous deletion of the entire gene. We confirmed this by means of our previously established quantitative fluorescent multiplex PCR (QFM-PCR) assay (Masson et al., 2007) (Fig. 1C). Walking QFM-PCR (Masson et al., 2007) was then employed to narrow down the deletion breakpoints, a strategy which led to the capture of the aberrant chromosomal junction by long-range PCR (Fig. 1D). Sequencing of the purified 3.5kb patient-specific product established the deletion as being 37,194bp in length, stretching from c.-28211 to c.*2066 (the first nucleotide of the translational initiation codon ATG is termed c.1 and the first nucleotide 3' of the translation stop codon is termed c.*1, in accordance with (den Dunnen et al., 2016)) or from chr5:147822595 to chr5:147859788 (hg38) (Fig. 1E). This deletion involved only the *SPINK1*gene.

In case 2, HRM analysis of the patient's genomic DNA showed that the first three amplicons of the four-exon *SPINK1* gene had similar melting curve patterns to those of normal controls but the fourth and last amplicon of the gene was absent (Fig. 2B). One possible reason for this latter finding would be the presence of an insertion within the target sequence in both alleles. We therefore increased the extension time of the PCR cycle from 30 to 45 seconds, and this yielded a single PCR product in the patient, ~400bp longer than its wild-type counterpart; this product was also present in both parents (Fig. 2C). Sequencing of this longer PCR

product revealed the insertion of an inverted full-length *Alu* sequence (precisely 359 bp in length) between nucleotides c.*14 and c.*15 in the 3'-untranslated region (3'-UTR) of the *SPINK1* gene (for further details of the mutation, see Supp. Fig. S3). The inserted sequence belongs to *Alu* Yb9, the youngest subfamily of *Alu* elements. Based upon an extensive literature search, we believe that this variant represents the first report of an *Alu* insertion into the 3'-UTR of a human gene causing genetic disease (Chen et al., 2006; Chen et al., 2005).

We analyzed the effect of the *Alu* insertion on *SPINK1* gene expression in the context of a full-length gene assay. Briefly, we cloned both the full length wild-type *SPINK1* genomic sequence (7217bp; extending from the translational initiation codon to c.*300) and its 7576 bp mutant counterpart into the pcDNA3.1/V5-His-TOPO vector. Reverse transcription-PCR (PT-PCR) of mRNAs from HEK293T transfected with the mutant construct did not yield any specific products; the 300bp RT-PCR product from HEK293T cells transfected with the wild-type construct (Fig. 2D) was confirmed by sequencing to be the wild-type *SPINK1* transcript. Moreover, we established long-term culture of Epstein-Barr virus-immortalized peripheral blood lymphocytes from the patient. RT-PCR analysis of mRNAs from the resulting cultured lymphocytes did not reveal any PCR products; by contrast, expression of the *SPINK1* gene was detected in the Epstein-Barr virus-immortalized lymphocytes from a healthy control (Fig. 2E).

To the best of our knowledge, no similar cases of severe infantile isolated EPI have ever been reported in the literature. Our two patients shared remarkably similar phenotypic manifestations, namely the occurrence of severe EPI from the age of five months, early appearance of diffusepancreatic lipomatosis (apparently at an age younger than five and three years, respectively), absence of significant abnormalities in any other organs, and normal growth and development upon enzyme replacement therapy. We adopted a reductionist approach to identify the genetic defect underlying this previously undescribed phenotypic profile. Thus, we first searched for mutations in *CFTR* and *SBDS*, the two primary causes of

severe EPI in children. When this search for causative mutations proved negative, we turned to the *PRSS1*, *SPINK1* and *CTRC* genes, mutations in which either cause or predispose to chronic pancreatitis. Two novel and non-identical homozygous mutations were identified in the *SPINK1*genes of the two patients.

SPINK1 encodes pancreatic secretory trypsin inhibitor, which has long been considered to be the first line of defense against prematurely activated trypsin within the pancreas (reviewed in (Chen and Férec, 2009)). A diverse range of loss of function mutations in the *SPINK1* gene have already been reported to cause/predispose to chronic pancreatitis (see http://www.pancreasgenetics.org/index.php). The most deleterious *SPINK1*genotype so far reported is consequent to homozygosity for c.194+2T>C. Based upon functional analysis performed using a full-length gene splicing assay (Zou et al., 2016) and RT-PCR analysis performed using gastric tissue from a c.194+2T>C homozygote (Kume et al., 2006), we surmise that ~10% of normal SPINK1 function would be present in the pancreas of a c.194+2T>C homozygote. Notably, c.194+2T>C homozygotes were reported to show extreme phenotypic variability, albeit with stones or calcification in the pancreas being common findings; the earliest onset of pancreatitis was reported to be at five years of age although none of the studied patients including a 75-year-old male, had developed severe EPI (Ota et al., 2010). The next most deleterious *SPINK1* genotypes, which include those heterozygous *SPINK1*variants that render the affected alleles completely nonfunctional (such as large deletions and frameshifting variants) have also been reported to cause chronic pancreatitis (Le Maréchal et al., 2004; Masson et al., 2006). By contrast, less deleterious *SPINK1*genotypes exemplified by the haplotype harboring the *SPINK1* c.101A>G (p.Asn34Ser) variant are generally considered to represent predisposing rather than a causative factors for chronic pancreatitis (Boulling et al., 2017). In short, *SPINK1* genotypes that do not result in a complete functional loss of the *SPINK1* gene tend either to predispose or to cause chronic pancreatitis.

In case 1, the functional consequence of the mutation identified, which removed the entire *SPINK1* gene but did not affect any other genes, is straightforward. In case 2, the mutation identified was an insertion of a full-length, inverted *Alu* element into the 3'-UTR of the *SPINK1* gene. We performed functional analysis using a full-length gene assay; the resulting complete loss of *SPINK1* expression was corroborated by RT-PCR analysis of mRNA from patient-derived cultured lymphocytes. The pathogenic relevance of two homozygous null mutations, involving a gene that plays an important role in pancreatic pathophysiology, and identified in two patients with severe infantile isolated EPI, is thus self-evident. In this regard, it is pertinent to relate our current findings to those obtained with *Spink3*-deficient (*Spink3*$^{-/-}$) mice (Ohmuraya et al., 2005) [murine *Spink3* and human *SPINK1* are orthologous]. In *Spink3*$^{-/-}$ mice, autophagic degradation of acinar cells occurred from day 16.5 after coitus; rapid onset of cell death occurred in the pancreas within a few days of birth, leading to death by the age of 14.5 days. Importantly, the massive amount of acinar cell death in *Spink3*$^{-/-}$ mice was associated neither with substantial infiltration of inflammatory cells nor with apoptosis (Ohmuraya et al., 2005). Whether or not the pancreatic damage in *Spink3*-deficient mice was trypsin-dependent (Ohmuraya et al., 2006) remains to be further investigated. Although physiological and pathological differences exist between human and mouse models, it is evident that a complete functional loss of the *SPINK1* gene and a partial functional loss of the *SPINK1* gene are associated with quite distinct clinical phenotypes.

There is an additional point to make. The parents of cases 1 and 2 were not subjected to genetic analysis but they should be obligate carriers of the respective variants. We do not possess laboratory test and imaging data with respect to the structure and function of the pancreas for any of the parents. As mentioned earlier, the father and paternal grandmother of case 2 had complained of recurrent abdominal pain, suggesting the existence of chronic pancreatitis; by contrast, the parents of case 1 were reportedly healthy. In this context, it is pertinent to mention that both heterozygous gain-of-function *PRSS1* missense variants (Khalid et al., 2006) and heterozygous loss-of-function *SPINK1* variants

have been reported to display variable penetrance (Le Maréchal et al., 2004). However, we consider it unlikely that the complete functional loss of the *SPINK1* gene such as we have described in the current study would manifest variable penetrance.

In summary, we have provided clinical and genetic evidence to define a new pediatric disease entity, namely severe infantile isolated EPI. Although the cases we describe were both homozygous for null *SPINK1* alleles, we might envisage that compound heterozygotes comprising two different null *SPINK1* alleles should have a same clinical phenotype. Finally, we would like to reiterate that the *Alu* insertion mutation identified here represents the first report of an *Alu* insertion into the 3'-UTR of a human gene causing genetic disease.

FIGURE LEGENDS

FIGURE 1 Identification of a homozygous deletion of the entire *SPINK1* gene in case 1. **A:** The patient's family pedigree. WT, wild-type. Del, deletion of the entire *SPINK1* gene. **B:** Magnetic resonance images of the patient's abdomen at five years of age: transverse sections centered on the body and tail of the pancreas acquired in T2-weighted image (left subpanel) and with Dixon fat suppression in-phase (right subpanel), respectively. The pancreas is indicated by an arrow. **C:** Confirmation of the homozygous deletion of the entire four-exon *SPINK1* gene in the patient by QFM-PCR. *EPHB6*, controlgene. **D:** The patient-specific long-range PCR product (approximately 3.5 kb). HC, healthy control. M, DNA marker. **E:** The sequence spanning the aberrant chromosomal junction; an 11 bp insertion was present between the two chromosomal ends. This *SPINK1* gene deletion was submitted to GenBank (https://www.ncbi.nlm.nih.gov/genbank/), its accession number being xxxx.

FIGURE 2 Identification and functional characterization of a homozygous *Alu* insertion within the 3'-UTR of the *SPINK1* gene in case 2. **A:** The patient's family pedigree. WT, wild-type. *Alu*, the *Alu* insertion mutation. **B:** Illustration of the non-amplification of the fourth and last exon of

the *SPINK1* gene in the patient (indicated by an arrow) under standard conditions of high-resolution DNA melting analysis. **C:** Detection of a longer PCR product in the patient (homozygous) and her parents (heterozygous) using a longer extension time to amplify exon 4 of the *SPINK1* gene. HC, healthy control. M, DNA marker. **D:** No detection of specific transcripts from HEK293T cells transfected with the *Alu* mutant construct in a full-length gene assay. The 1000-bp band appears to be artefactual because it was also detected in cells transfected with the empty pcDNA3.1 vector (EV). The 300-bp band was confirmed by DNA sequencing to correspond to the wild-type *SPINK1* transcript. NC, negative control. **E:** RT-PCR data from cultured lymphocytes of a healthy control (HC) and the patient. The variant was submitted to GenBank, its accession number being xxxx.

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