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A novel *EXT2* mutation in a consanguineous family with severe developmental delay, microcephaly, seizures, feeding difficulties, and osteopenia extends the phenotypic spectrum of autosomal recessive *EXT2*-related syndrome (AREXT2)

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ABSTRACT

Keywords:

Intellectual deficiency
Seizures
Recessive
EXT2
Mutation
AREXT2
Homozygous

We report a consanguineous family where 2 boys presented with developmental delay, hypotonia, microcephaly, seizures, gastro-intestinal abnormalities, osteopenia, and neurological regression. Whole exome sequencing performed in one of the boys revealed the presence of a novel homozygous missense variant in the *EXT2* gene: c.11C > T (p.Ser4Leu). Segregation analysis by Sanger sequencing confirmed homozygous by descent autosomal recessive transmission of this mutation. Another family was previously reported with homozygous mutations in this gene in four siblings affected with a nearly similar clinical condition (Farhan et al., 2015). We discuss the similarities and differences between the two syndromes and propose AREXT2 as a new acronym for *EXT2*-related diseases.

1. Introduction

Mutations in the *EXT2* gene (Exostosin Glycosyltransferase 2) are known to cause an autosomal dominant disorder characterized by multiple exostoses that appear at early childhood (MIM 133701). Recently, two homozygous missense mutations in the *EXT2* gene (p.Met87Arg and p. Arg95Cys, associated *in cis* on the same allele) were found in four sibs from the same Mennonite family affected with severe developmental delay (DD), hypotonia, dysmorphic facial features, seizures and scoliosis, described as the SSM (Seizures, Scoliosis, and Macrocephaly) syndrome (Farhan et al., 2015) (MIM 616682).

EXT2 encodes a glycosyltransferase involved in the synthesis of heparan sulfate (HS), a glycosaminoglycan that is covalently attached to proteoglycan core proteins in proteoglycans (PG), and known to be implicated in many cellular functions by interacting with protein ligands, including a wide variety of growth factors and morphogens (Bishop et al., 2007; Gorski and Stringer, 2007; Kreuger et al., 2006). Together with *EXT1* (Exostosin Glycosyltransferase 1), *EXT2* forms a stable complex in the Golgi apparatus (McCormick et al., 2000), which assembles HS chains using nucleotide sugars imported from the

cytoplasm (Esko and Selleck, 2002).

Here, we report on a first-degree consanguineous Syrian family, where 2 sibs present a severe syndrome characterized by developmental delay, microcephaly, seizures, gastro-intestinal problems, and osteopenia, for whom we identified a new homozygous missense mutation in the *EXT2* gene: c.11C > T (p.Ser4Leu).

2. Materials and methods

This study is conformed to the tenets of the Declaration of Helsinki.

2.1. Patients

Patients II.2 and II.3 (Fig. 1) are two brothers, born to healthy first-cousin Syrian parents. They had an older sister (II.1) who died at the age of 7. Both affected siblings underwent extensive work-up and routine blood tests.

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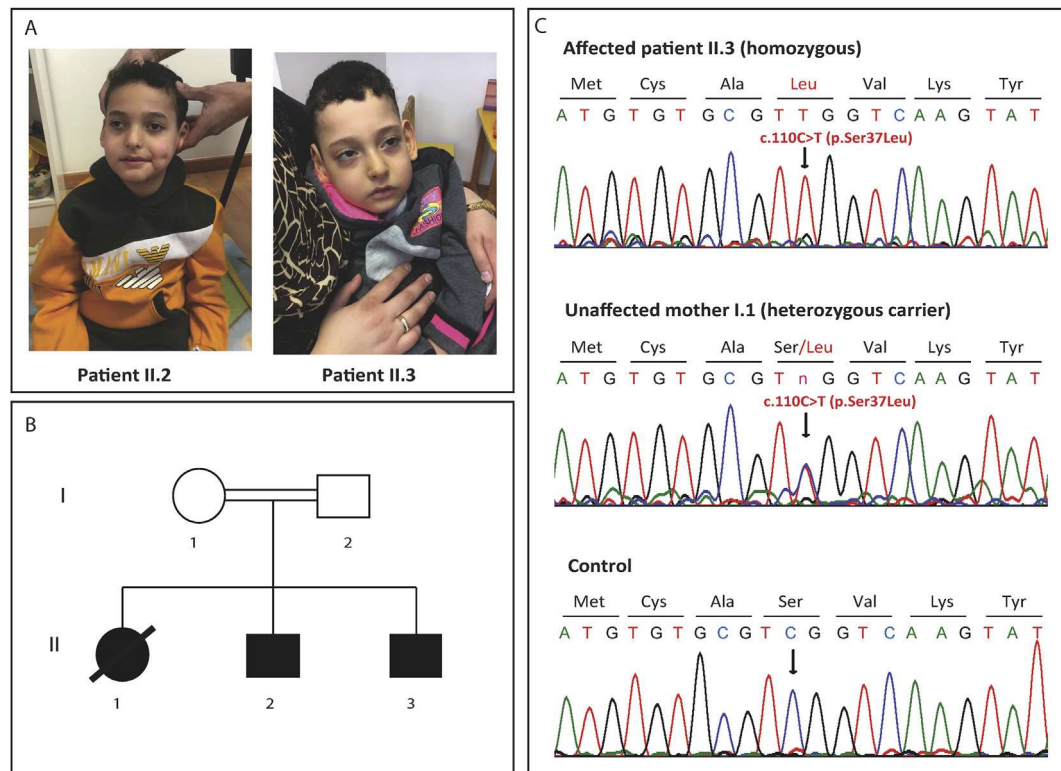


Fig. 1. (A) Photographs of patients: II.2 (left) and II.3 (right). (B) Pedigree of the studied family. Blackened symbols indicate affected individuals. (C) Chromatograms from one patient (II.3), one parent (I.1) and a control showing the homozygous missense mutation c.110C > T (p.Ser4Leu) in *EXT2* exon 2 (NM_207122), in the family.

2.2. Samples

After informed consent was obtained from all individuals included in this study, EDTA blood samples were collected, and genomic DNA was extracted from lymphocytes with the use of standard methods. All protocols performed in this study complied with the ethics guidelines of the institutions involved.

2.3. Whole exome sequencing (WES)

Paired-end whole exome sequencing was carried out on patient II.2 (Fig. 1) using Nextera Rapid Capture Exome (Illumina, San Diego, USA) following the manufacturer's recommendations.

Raw data were mapped to the built of the human genome (hg19) by using BWA 0.7.5 (Li and Durbin, 2009). Variant calling was subsequently performed using GATK (McKenna et al., 2010) and annotation was carried out with ANNOVAR (Wang et al., 2010). All subsequent steps were performed using our *in-house* software for variant annotation and segregation VarAFT (Desvignes et al., 2018). Given the consanguinity between the parents and the apparent autosomal recessive transmission mode, we analyzed the WES data from patient II.2 searching, in the first place, for homozygous mutations. This analysis was performed in a two-phased approach: (i) targeted analysis of the WES data on 2 different lists of genes: a list of 714 genes known to be implicated in neurological diseases and a list of 287 genes known to be implicated in different forms of epilepsy (Supplementary Table 1 and 2), and ii) analysis of the WES data. In order to refine the obtained lists of candidate nucleotide variations, filtering was performed on the Genome Aggregation Database (gnomAD, <http://gnomad.broadinstitute.org/>): i.e., all variants with a frequency above 1% in this dataset were removed. Additional filtering was also performed using two *in-house* exome databases: i) a database containing the WES data from 39 Lebanese and Syrian healthy related indi

viduals from 339 Lebanese and Syrian individuals (including patients). In order to predict the deleterious effect of the identified sequence variations, different bioinformatics tools were applied; such as MutationTaster (<http://www.mutationtaster.org/>) (Schwarz et al., 2010), SIFT (<http://sift.bii.a-star.edu.sg/>) (Kumar et al., 2009), PROVEAN (<http://provean.jcvi.org/index.php/>) (Choi et al., 2012), PolyPhen-2 (<http://genetics.bwh.harvard.edu/pph2/>) (Adzhubei et al., 2010), UMD predictor (<http://umd-predictor.eu/>) (Salgado et al., 2016) and CADD (Kircher et al., 2014).

2.4. Sanger sequencing

The c.110C > T (p.Ser4Leu) variant in exon 2 of *EXT2* (NM_207122), identified by WES sequencing in patient II.2, was tested by Sanger sequencing of PCR amplified fragments in family members, for whom DNA was available: patients II.2 and II.3; mother (I.1) and father (I.2). The variation was segregated according to autosomal recessive inheritance. Genomic and cDNA sequences of *EXT2* (Genbank accession number: NM_207122) were obtained from the UCSC Genomic Browser, February 2009 human reference sequence (GRCh37/hg19). Primers used for PCR amplification were designed using Primer3 (<http://frodo.wi.mit.edu>) (sequences available upon request). PCR products were purified by mixing with an equal volume (10µl) of AMPure beads (Beckman Coulter, USA) according to the manufacturer's instructions. Both strands were sequenced using the Big Dye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). Sequence reactions were purified on Sephadex G50 (Amersham Pharmacia Biotech, Foster City, CA) and capillary electrophoresis was performed on Genetic Analyser 3130x (Applied Biosystems). Electrophoregrams were analyzed using the Sequence Analysis Software version 5.2 (Applied Biosystems) and aligned with the reference sequence using Sequencher v5.4.6 (Genecodes, USA).

3. Results

3.1. Clinical report

For both affected sibs (II.2 and II.3, Fig. 1), delivery was normal and at term with no recall of exposure to pre- or perinatal environmental toxins. Birth head circumferences were not reported but considered in the normal limits by the parents. Weights at birth were 3600 g (75th percentile) and 4000 g (95th percentile), and length birth were 50 cm (60th percentile) and 52 cm (90th percentile) respectively.

Psychomotor delay was noted for both at the age of 3 months. The eldest boy (II.2) could sit alone at the age of 18 months while his affected brother (II.3) never could. Both never walked, even when helped, and never talked. Seizures appeared at the age of 16 months (Patient II.2) and 6 months (Patient II.3). Epileptic crises consisted of atonic seizures, complex partial seizures and possible brief tonic seizures for the eldest boy, and clusters of epileptic spasms for the youngest. Different anti-epileptic drugs were tested but without success. Both affected sibs had many episodes of gastroesophageal reflux, and feeding difficulties. The youngest boy (II.3) had to be fed by gastrostomy and complained from chronic constipation. Heart and genital exams were normal.

When they were brought to us for consultation, patient II.2 was 8 years old. He was hypotonic but could stay seated without help. He was responding randomly to the orders but was speechless. His weight was 22 Kg (10th percentile), his length 126 cm (75th percentile) and his OFC 49.5 cm (3rd percentile). A clinical examination noted an axial hypotonia, a flat forehead, large ears, decayed teeth, and a gingival hypertrophy. Electroencephalogram (EEG) showed epileptiform discharges of the type seen in the setting of a symptomatic generalized epilepsy. Video-EEG exam for both patients revealed absence of normal sleep activity during behavioral sleep, multifocal independent epileptiform discharges and burst of paroxysmal fast activity, which were sometimes associated with brief tonic elevation of the eyes and staring during the awake state.

His affected brother II.3 was seen at the same moment. He was 5 years and 10 months old. He was more deeply affected than his brother. At examination, his weight was 16 Kg (5th percentile), his length 119 cm (75th percentile) and his OFC 45 cm (< 3rd percentile). He had the same features seen in his brother but gingival hypertrophy was more important and he had a single palmar crease on the left hand. EEG and video-EEG were in favor of epileptic spasms, modified hypsarrhythmia with a final diagnosis of West syndrome.

Brain MRI, and abdominal and pelvic ultrasound exams were normal for both sibs, while bone density test revealed the presence of osteopenia. Family history noticed a possible affected sister who died at age 7 from respiratory distress. According to the parents she had exactly the same clinical course as her 2 affected brothers; mainly, severe developmental delay, severe intellectual disability (ID), no speech, feeding difficulties, constipation, and seizures.

3.2. Laboratory studies

Results of complete blood counts, serum electrolytes, blood glucose levels, triglycerides, cholesterol, amino acid studies of plasma and urine, urinary screening for organic acids, urinalysis, thyroid, liver and renal function tests, CPK, Ca, P, were all unremarkable. Lactic and pyruvate acids were slightly elevated. Karyotypes were normal.

3.3. Genetic analysis

Targeted analysis of the WES data from patient II. 2 (mean coverage depth of 79X), on the two different lists of genes involved in neurological diseases and epilepsy (Supplementary Table 1 and 2), did not allow to identify any candidate variant in the disease, following autosomal recessive or X-linked transmission. In consequence, we searched

for homozygous variants in the WES sequence data from patient II.2. This led to the identification of 43110 variants, of which 66 were located in the coding sequences of genes or at splice sites, and had a frequency below 1% in the gnomAD dataset (<http://gnomad.broadinstitute.org/>). Further filtering on our local datasets allowed narrowing down the number of candidate variants to 49. By removing non-synonymous variants, we ended up with a final list of 25 candidate variants (Supplementary Table 3). This final list was then further refined and prioritized based on several criteria: allele frequencies above 1% in some gnomAD dataset subpopulations, homozygosity in the gnomAD dataset, function of the affected genes and implication of these genes in diseases. We finally propose the homozygous missense variation c.11C > T (p.Ser4Leu) in exon 2 of *EXT2* (NM_207122) as the possible causative mutation in the disease affecting our patients (Clinvar Accession Number: SCV000783102), considering the following: i) a very low allele frequency in the gnomAD dataset (6.908e-05), ii) a prediction for pathogenicity by several prediction tools (UMD-Predictor, SIFT, Polyphen, Mutation Taster, LRT, Provean, CADD), and iii) the previous description of mutations in this gene in four sibs from one consanguineous Mennonite family affected with a close clinical condition described in OMIM as the SSMS syndrome (Seizures, Scoliosis, and Macrocephaly Syndrome, MIM 616682) (Farhan et al., 2015). The variant was well sequenced by WES in patient II.2 (94X depth of sequencing) and segregation analysis by fluorescent Sanger sequencing confirmed homozygosity by descent for this variant in the family (patients II.2 and II.3 are homozygous and both parents are heterozygous, Fig. 1). The Serine at position 4 of the amino acid sequence is highly conserved among vertebrates and is located upstream of the two functional domains of the protein: the exostosin domain (aa 100–380) and the glycosyl transferase 64 domain (aa 456–697) (Fig. 2).

4. Discussion

Here, we report a Syrian family where 2 boys present features consisting of severe DD and ID, hypotonia, microcephaly, feeding difficulties, gastroesophageal reflux, constipation, seizures, osteopenia, and neurological regression. Through WES sequencing, we identified a novel homozygous c.11C > T (p.Ser4Leu) missense variation in exon 2 of the *EXT2* gene (NM_207122); as the culprit mutation in the disease affecting the two siblings. The mutation is located at the N-terminal end of the protein, upstream of the exostosin domain and is highly conserved among species.

In humans, heterozygous mutations in *EXT2* cause Hereditary Multiple Exostoses (HME) type II (MIM 133701), an autosomal dominant disease characterized by cartilaginous/bony tumors (called osteochondromas or exostoses), that form within perichondrium in long bones, ribs and other elements (Pacifci, 2018). HME, also called Multiple Osteochondromas Syndrome (MO) is caused by mutations in both *EXT1* and *EXT2*, which together with *EXTL1* (Wise et al., 1997), *EXTL2* (Wuys et al., 1997) and *EXTL3* (Van Hul et al., 1998), encode glycosyltransferases of the exostosin family (EXTs) (Busse-Wicher et al., 2014). EXT proteins are glycosyltransferases involved in the adhesion and/or polymerization of heparan sulfate (HS) chains at HS proteoglycans (HSPG) (Esko and Selleck, 2002) and are characterized by their homology in the carboxyterminal region, and their ubiquitous expression pattern (Van Hul et al., 1998).

Recently, two homozygous missense mutations, p. Met87Arg and p. Arg95Cys, associated *in cis* on the same allele, were found in the *EXT2* gene; in four sibs from a consanguineous Mennonite family, affected with severe developmental delay, hypotonia, coarse facies with hypertelorism, poor speech, seizures, macrocephaly, and scoliosis, designated as Seizures-Scoliosis-Macrocephaly syndrome (SSM, MIM 616682) (Farhan et al., 2015). Despite common clinical features between the patients from this study and those described by Farhan et al. (2015), some differences were noted (Table 1). The present patients have a more severe clinical presentation. For instance, DD, ID,

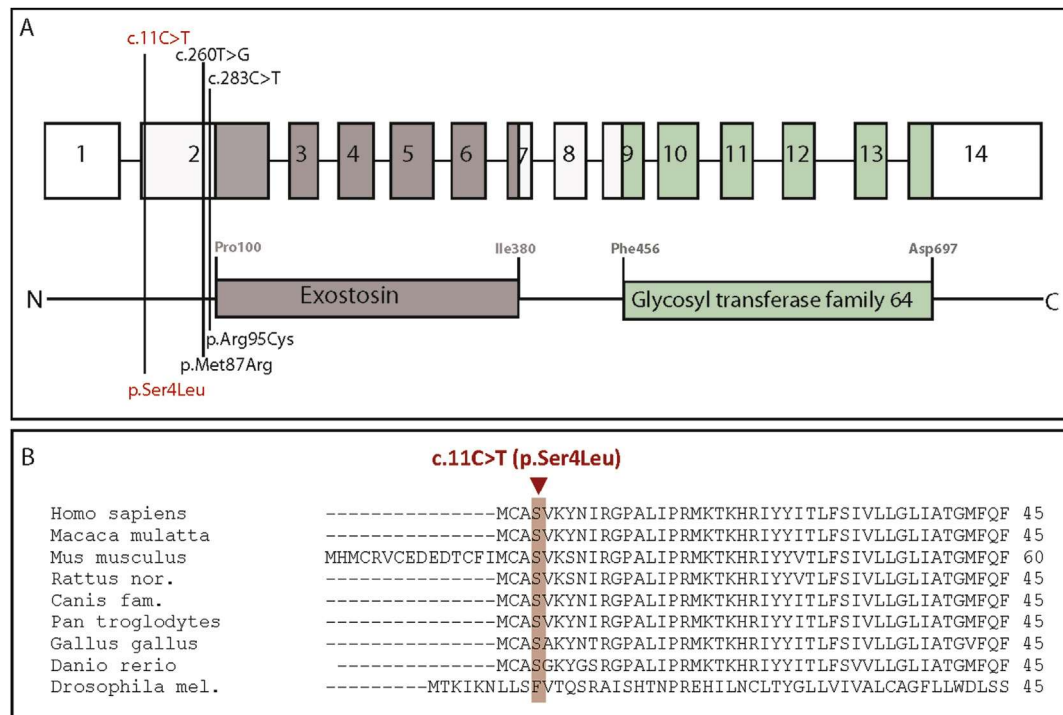


Fig. 2. (A) Schematic representation of the *EXT2* gene. Top: structure of the *EXT2* gene, with exons displayed as blocks, Bottom: *EXT2* protein with two functional domains. Mutations identified in this study and by Farhan et al. (2015) are indicated in red and black, respectively. (B) Amino acid sequences of *EXT2* from the indicated species were aligned using the program CLUSTAL 2.1. Shown is the protein region comprising the mutation under investigation. Serine 4 (human numbering) highlighted in red is highly conserved across vertebrates. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

hypotonia, speech problems, and course of the disease are much more severe, and seizures appeared at an earlier age. No scoliosis was noted in the present cases (although the patients are still young). The gingival hypertrophy seen in the 2 brothers might be secondary to the anti-epileptic treatments. The main difference between the patients from our

family and the family reported by Farhan et al. (2015), is the head circumference. In this report, the patients have post-natal microcephaly while the patients reported by Farhan et al. (2015), had a macrocephaly; but whether or not this last trait is familial is questionable. Farhan et al. named the entity that they reported with the acronym SSM

Table 1
Clinical comparison between the patients from this study and those described by Farhan et al.2015.

Clinical features	Affected individuals					
	Farhan et al.,2015 Case 1	Farhan et al.,2015 Case 2	Farhan et al.,2015 Case 3	Farhan et al.,2015 Case 4	The present study Patient II.2	The present study Patient II.3
Sex	M	M	F	M	M	M
Birth weight	70th P	78th P	78th P	88th P	75th P	95th P
Birth length	n.a	n.a	> 95th P	n.a	60th P	90th P
Birth OFC	n.a	n.a	n.a	n.a	n.a	n.a
Age at examination (y)	19	14	11	10	8	6
Stature	75th P	10th P	n.a	50th P	75th P	75th P
Head circumference	> 98th P	90th P	n.a	98th P	3rd P	< 3rd P
Hypotonia	+	+	+	+	+	++
Developmental delay	+	+	+	+	++	+++
Regression	+	n.a	n.a	+	+	+
Intellectual disabilities	+	+	+	+	++	+++
Speech	Rudimentary	Rudimentary	Rudimentary	Rudimentary	Absent	Absent
Seizures	+	+	+	+	+	+
Hypertelorism	+	-	n.a	n.a	-	-
Gingival hypertrophy	n.a	n.a	n.a	n.a	+	+
Cryptorchidism	+	+	n.a	+	-	-
Cardiovascular anomalies	+	n.a	+	-	-	-
Gastroesophageal reflux	+	+	+	n.a	+	+
Feeding problems	+	n.a	Tube-fed	n.a	+	Tube-fed
Constipation	+	n.a	n.a	n.a	-	+
Skin anomalies	+	+	+	+	-	-
Low bone density	+	n.a	+	+	+	+
Scoliosis	+	+	+	n.a	-	-

+, present; -, absent; n.a., not available; P, percentile.

for Seizures-Scoliosis-Macrocephaly (Farhan et al., 2015). If one considers that both families have the same disorder, thus the acronym must be changed and we propose to use AREXT2 (Autosomal Recessive EXT2-related syndrome) instead.

After Farhan et al. (2015), our study is the second report involving *EXT2* mutations in a disease with no exostoses. Although unexpected, the absence of exostoses is not surprising. Indeed, as in the patients described by Farhan et al. (2015), the novel mutation that we describe here is a missense mutation (p.Ser4Leu), laying at the N-terminal part of the protein, outside the two conserved functional domains of the *EXT2* protein: the exostosin domain (aa 100–380) and the glycosyl transferase 64 domain (aa 456–697) (Fig. 2). Moreover, it is well known that inactivating mutations (nonsense, frameshift, and splice-site mutations) in *EXT1* and *EXT2* represent the majority of HME causing mutations (75–80%) (Jennes et al., 2009) (<https://databases.lovd.nl/shared/variants/EXT2>) and result in a premature termination of the *EXT* protein translation and loss of function, whereas missense mutations are less common and result in loss of *EXT* activity (Jennes et al., 2009; Pacifici, 2017; Wuyts and Van Hul, 2000).

The mechanisms underlying the formation of exostoses is yet unknown, but it has been suggested that it occurs when Heparan Sulfate (HS) synthesis dips below a certain threshold (Roehl and Pacifici, 2010). In conditional mutant mouse models, it has been shown that complete loss of HS is required to induce osteochondroma formation while low amounts of HS are sufficient to maintain the growth plate structure (Piombo et al., 2018). Additionally, it is well known that *EXT2* has significantly lower enzymatic activity than *EXT1* and may be more important during formation and transport of the HS polymerase complex than in HS synthesis (Roehl and Pacifici, 2010). In this study, both affected sibs and their parents (heterozygous for the mutation) have normal bones on x-rays and a normal stature suggesting that residual amounts of HS synthesized by *EXT1* and *EXT2* are sufficient to prevent the development of exostoses/osteochondromas.

Disorder of the biosynthesis of HS causes impairment of cellular function and abnormal morphogenesis, which could lead to many diseases (Nadanaka and Kitagawa, 2008). In fact, HS chains bind many proteins such as the members of the fibroblast growth factor (FGF) family and their receptor tyrosine kinases, transforming growth factors (TGFs), bone morphogenetic proteins (BMPs), Wnt proteins, chemokines and interleukins, as well as enzymes and enzyme inhibitors, lipases and apolipoproteins, and ECM and plasma proteins (Bishop et al., 2007). In order to study mechanisms promoted by protein-HSPG interaction, knockout and transgenic mice have been generated, often with surprising phenotypes, and evidenced the role of HSPG in metabolism, transport, information transfer, support and regulation at the systemic level, as well as the cellular level reviewed in (Bishop et al., 2007). Alzheimer and Parkinson's diseases are examples of HS' implication in neurodegenerative disorders; in the first one, HSPG play a role in amyloid b-peptide plaque pathogenesis (Verdier et al., 2004) and in the second, a HSPG binds to a-synuclein and enhances its insolubility and the formation of aggregates characteristics of the disease (Liu et al., 2005).

Another example supporting the phenotypic variability observed in patients affected with mutations in an *EXT* gene is a recent study where four homozygous missense mutations in *EXTL3* were identified in nine individuals from five unrelated families affected by neuro-immuno-skeletal dysplasia syndrome (Oud et al., 2017). Taken together, all these results support the implication of *EXT2* mutation in the developmental syndrome affecting our patients.

Indeed, many knock-down studies demonstrated the role of HS in developmental pathways and more precisely those implicated in neuronal development (Fischer et al., 2011; Nurcombe et al., 1993; Yamada et al., 2017). The heparin-binding fibroblast growth factors FGF-1 and FGF-2, whose activities are regulated by HSPG, act as potent developmental regulators of the proliferation, migration, and differentiation of murine neural precursor cells (Nurcombe et al., 1993).

Furthermore, *ext2* was shown to enhance fgf and wnt signaling in zebrafish and impaired brain patterning was described in *ext2* mutants where the formation of fewer signaling fgf receptor dimers was thought to be the result of reduced levels of HS (Fischer et al., 2011).

Therefore, we hypothesize that the p. Ser4Leu missense mutation described in our patients may also lead to a partial loss of protein's activity disrupting HS synthesis. Consequently, residual levels of *EXT2* in our patients' cells are not sufficient for maintaining normal cellular functions, among others neural development, causing our patients' phenotype. Our hypothesis is in agreement with the one suggested by Farhan et al., which states that *EXT2* homozygous mutations in human, could disrupt developmental cascades requiring *EXT2* for propagation, such as FGF and Wnt signaling, as was previously demonstrated in *Ext2*-knockdown studies (Fischer et al., 2011; Norton et al., 2005; Nurcombe et al., 1993). Another possibility is that, in our patients, the residual expression of mutated *EXT2* is disrupting interactions with partners important for global development, such as NDST1. Indeed, in SSM patients' fibroblasts, *EXT2* is partially expressed and active but the levels of *NDST1* are reduced (Farhan et al., 2015) and bi-allelic mutations in *NDST1* have been recently described in girl with a syndrome sharing many clinical features with SSM patients and our patients (Armstrong et al., 2017): cranial nerves dysfunction, gastroesophageal reflux, history of a seizure, ataxia, developmental delays, head sparing failure to thrive, and minor malformations including distinctive facial features and a bifid uvula.

In conclusion, we report on a second family with a homozygous mutation in the *EXT2* gene presenting with Intellectual Disability/Developmental Disability (ID/DD), micro/macrocephaly, absent to rudimentary speech, gastro-intestinal abnormalities, seizures, and osteopenia, that we propose to name AREXT2 (Autosomal Recessive *EXT2*-related syndrome). Our results suggest that AREXT2 is due to a defect in Heparan Sulfate synthesis involving *EXT2* and *NDST1*. Given the rarity of this syndrome, the report of novel cases is important to better delineate the clinical spectrum associated with this gene. The common place use of WES in the clinical diagnoses of rare neurodevelopmental disorders should uncover more cases, particularly in countries in which consanguinity is highly prevalent.

Conflicts of interest

None.

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