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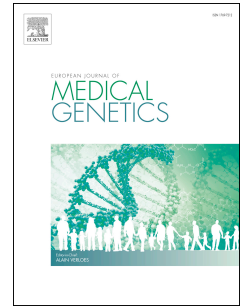
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Two females with mutations in *USP9X* highlight the variable expressivity of the intellectual disability syndrome

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Two females with mutations in *USP9X* highlight the variable expressivity of the intellectual disability syndrome

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Abstract

The genetic causes of intellectual disability (ID) are heterogeneous and include both chromosomal and monogenic etiologies. The X-chromosome is known to contain many ID-related genes and males show a marked predominance for intellectual disability. Here we report two females with syndromic intellectual disability. The first individual was relatively mild in her presentation with mild-moderate intellectual disability, hydronephrosis and altered pigmentation along the lines of Blaschko without additional congenital anomalies. A second female presented shortly after birth with dysmorphic facial features, post-axial polydactyly and, on follow-up assessment, demonstrated moderate intellectual disability. Chromosomal studies for Individual 1 identified an X-chromosome deletion due to a *de novo* pericentric inversion; the inversion breakpoint was associated with deletion of the 5'UTR of the *USP9X*, a gene which has been implicated in a syndromic intellectual disability affecting females. The second individual had a *de novo* frameshift mutation detected by whole-exome sequencing that was predicted to be deleterious, NM_001039590.2 (*USP9X*): c.4104_4105del (p.(Arg1368Serfs*2)).

Haploinsufficiency of *USP9X* in females has been associated with ID and congenital malformations that include heart defects, scoliosis, dental abnormalities, anal atresia, polydactyly, Dandy Walker malformation and hypoplastic corpus callosum. The extent of the congenital malformations observed in Individual 1 was less striking than Individual 2 and other individuals previously reported in the literature, and suggests that *USP9X* mutations in females can have a wider spectrum of presentation than previously appreciated.

Keywords: *USP9X*, Intellectual disability, Lines of Blaschko

1. Introduction

Intellectual disability (ID) affects 1-2% of the population (Leonard and Wen 2002; Ropers 2010) and can be due to genetic and/or environmental causes. Approximately 20% of children with ID have a detectable cytogenetic alteration seen on chromosomal microarray (Miller et al. 2010). The individuals with ID attributable to the presence of a copy-number variant (CNV) can be syndromic in their presentation. The deletions and duplications can encompass several genes, and the phenotype may be due to the actions of one or more genes present within the region. Intellectual disability can also be monogenic. For both CNV-related and monogenic ID, there is a predominance of males due to the presence of critical genes for neurodevelopment on the X-chromosome. Males with hemizygous mutations in these X-linked genes tend to be more severely affected, with female carriers of X-linked mutations frequently being unaffected or demonstrating milder manifestations of disease. Haemophilia A is the classic example where female carriers of X-linked mutations affecting *F8* are relatively asymptomatic (Plug et al. 2006).

Missense mutations in *USP9X* are recognized to cause a non-syndromic intellectual disability in males (Homan et al. 2014). However, in females, mutations in *USP9X* have been identified as responsible for a clinically recognizable intellectual disability syndrome that is associated with short stature, anal anomalies, polydactyly, heart defects, cleft palate and structural brain malformations (Brett et al. 2014; Reijnders et al. 2016). The mutations are typically loss of function in the females and the additional clinical findings are not present in males carrying missense mutations. Therefore, while ID is present in both males and females, the overall

phenotype appears quite different in females, particularly as females often present with congenital malformations which are not typically seen in males.

Here we present two females with *USP9X*-related ID. The first female individual has a deletion in the 5'UTR of *USP9X* due to a *de novo* X chromosome rearrangement. Her mutation is not the typical loss of function type mutation in *USP9X* previously reported in females with syndromic ID associated with malformations (Reijnders et al. 2016). Her relatively milder presentation broadens the spectrum that can be associated with *USP9X* mutations in females, and suggests that mutations that are not clearly loss of function can still lead to syndromic ID in females. The second female shows a more typical presentation of the *USP9X* syndrome in females, due to a novel frameshift mutation. These two cases highlight the spectrum of the *USP9X* clinical presentation.

2. Material and Methods

Individual 1: A standard karyotype (G-banding) was performed as part of routine work-up at our center to assess for chromosomal rearrangements in amniocytes and parental samples for Individual 1. A commercially available comparative genomic hybridization array test comprising 400,000 oligonucleotide probes and single nucleotide polymorphisms (Baylor College of Medicine- Medical Genetics Laboratories, Houston, United States) was also performed on amniocytes after the karyotype results. The X-inactivation patterns for both Individual 1 and 2 was determined by assessing the methylation pattern of a polymorphic repeat locus containing a methylation-sensitive restriction enzyme (HpaII) site within the promoter region of the *SLITRK4* gene at Xq27.3 using methylation-sensitive PCR (Bertelsen et al. 2011). In brief, HpaII digested and mock digested genomic DNA from the individual's lymphocytes

were PCR amplified. The PCR products were analyzed on an ABI 3130 XL genetic analyzer (Applied Biosystems) and the X-inactivation ratios were calculated as previously described (Lau et al. 1997).

Individual 2: This child was most recently assessed through the Undiagnosed Diseases Program-Western Australia, a cross-disciplinary public health service informed by the Undiagnosed Diseases Program at the United States National Institutes of Health (Tift and Adams 2014) and operating as part of the Undiagnosed Diseases Network International (Taruscio et al. 2015). After review at the clinical panel meeting, whole exome sequencing was initiated and clinical exome data was analysed, as reported previously (Baynam et al. 2016).

3. Clinical presentation and results

Individual 1: The healthy, 39 year old G5P2 mother of the first female presented to the Prenatal Genetics Clinic for evaluation of fetal renal pyelectasis and a single umbilical artery at 17 weeks gestation. Rapid aneuploidy detection (RAD) was performed with subsequent karyotype on amniocentesis. The RAD was normal, however the karyotype showed an apparent pericentric inversion on the X chromosome; 46, X,inv(X)(p11.4;q21). Karyotypes performed on parental cultured lymphocytes showed this was a *de novo* pericentric inversion in the fetus. As a result of the *de novo* chromosomal abnormality, a prenatal microarray was offered and showed a small loss of 0.326 Mb on the X chromosome, arr (hg19) Xp11.4 (40644087-40970033) x1, which deletes the 5'UTR of *USP9X* (Figure 1). The pregnancy was otherwise unremarkable aside from maternal hypothyroidism for which the mother took L-thyroxine. There were no exposures to teratogens. The baby was delivered by spontaneous vaginal delivery at 38 weeks and 5 days. Birthweight was 2.79 kg (50th percentile) and head circumference and length were not recorded.

There were no dysmorphic features or congenital malformations recorded at birth with the exception of the 2-vessel cord. The prenatal diagnosis of pyelectasis prompted a renal ultrasound that showed bilateral grade 2 hydronephrosis with a normal voiding cystourethrogram.

The child's early life was unremarkable with the exception of a poor weight gain that responded well to formula supplementation. During her assessment for poor growth, she was noted to have mild hypotonia and a head ultrasound showed prominent lateral, third and fourth ventricles. A subsequent MRI revealed prominent extra-axial spaces but no cortical malformation (Figure 2). At 6 months of age she was noted to have hyper and hypopigmented streaks following the lines of Blaschko on her torso and back. There was no history of intellectual disability, developmental delays or seizures in her family. There was no history of consanguinity.

The child presented again to the Medical Genetics Clinic at 10 months of age for developmental delay. At that time her head circumference was 43 cm (10th percentile) with a weight of 6.4kg (0.1-3rd percentile) and a length of 67cm (3rd percentile). She was described as non-dysmorphic. A Woods lamp examination showed lines of Blaschko to her torso.

A follow-up assessment at 3 years of age showed a well appearing female without striking dysmorphisms. There were no health concerns with the exception of delays in her development. Her first independent steps were at 2 years and 9 months of age. Her first single words were at 2 years of age. At age 3 years of age she had 3 to 5 single words only with no short phrases. There was no history of regression and no abnormal movements or seizures. A review of systems was unremarkable. Her height was 92cm (10th percentile), weight was 12.1kg (5th percentile), and head circumference was 49cm (25th percentile). Head was normally shaped and she did not have

a broad forehead. Palpebral fissures were not up- or down slanted. Ears had normal structure, attached lobules, and were not posteriorly rotated or low set. Palate and uvula were normally formed although her mouth was down-turned. She had dental caries and widely spaced teeth. While she was not considered dysmorphic, her teeth and down-turned mouth did give her a distinctive appearance. She was proportionate with no asymmetry in her face, chest or limbs. Her spine was straight with no sacral dimple. There was no hypertrichosis. There was hypermobility in her small joints, her hands and feet were slender and there was hyperpigmentation along the lines of Blaschko. The remainder of her examination was unremarkable.

X-inactivation studies were performed for individual 1 after review of her microdeletion indicated that the recently described USP9X gene was impacted. These studies showed a preferential skewing of 80:20 of the X-chromosome from lymphocytes.

Individual 2: The second individual was born to a healthy 37 year old G1P0 mother. The first trimester screen was high-risk (1/130) for Trisomy 21 and an amniocentesis was performed with a normal G-banded karyotype at a 550 band level. The delivery was induced for growth deceleration. She was born at 38 weeks gestation and here Apgar scores were 6 at 1 minute and 9 at 5 minutes. Birthweight was 2.5 kg (10th percentile), length was 47.5 cm (25th percentile) and head circumference was 32 cm (10th percentile). At birth she was found to have a two-vessel cord, post-axial polydactyly (non-articulated tissue on the right hand), a prominent facial hemangioma and she was dyspneic. An abdominal ultrasound at 8 days of age was normal. An echocardiogram showed dilated cardiomyopathy with pulmonary hypertension that resolved without intervention. An MRI at 15 months of age showed moderate ventriculomegaly with

mild to moderate dilatation of the third and fourth ventricles with bowing of the corpus callosum (Figure 3). A chromosomal microarray at 9 years of age was normal.

During follow-up assessments she was noted to have recurrent ear infections, high-frequency hearing loss, mild myopia, laryngomalacia and global developmental delay. Her first independent steps were at 4 years. At 9 years of age she had single words and for the most part communicated by sign language. Her receptive speech exceeded her expressive speech. There was no history of regression.

At 9 years of age her height was 132cm (25th percentile), weight was 24.6 kg (10th percentile), and head circumference was 55cm (98th percentile). She had a tall, broad forehead. She had a marked facial hemangioma to her glabella and forehead. Palpebral fissures appeared short (not measured) and there was a wide inner canthal distance. She had a wide, flat nasal root with flared nares. Her philtrum was long, with a tented upper lip and a thick lower lip vermilion. Her dentition was crowded and her palate was high arched with no bifid uvula. She had midfacial flattening and micro-retrognathia. Her ears were low set and posteriorly rotated with attached lobules. She had facial asymmetry, and asymmetric pubertal development with a breast bud on the left and axillary hair on the right. Limbs were symmetric and proportionate. She had a thoracic scoliosis and a pectus excavatum. She had a sandal gap between first and second toes, mild 2,3 cutaneous toe syndactyly and long fingers and toes. There was no hypertrichosis. The remainder of her examination was unremarkable. Her facial features over time are illustrated in Figure 3.

WES identified a frameshift mutation detected that was predicted to be deleterious, NM_00103959.2 (*USP9X*): c.4104_4105del (p.(Arg1368Serfs*2)). Sanger sequencing was

performed and showed the mutation was *de novo*. The mutation has not been previously seen in ExAc or ClinVar databases. No loss of function mutations have been reported in the ExAC browser database. X-inactivation studies of lymphocytes from Individual 2 showed no evidence of significant, or preferential skewing of the X chromosome. The frameshift mutation and the chromosomal deletion observed in Individual 1 were submitted to the ClinVar database (SUB2523087).

4. Discussion

A recognizable, syndromic form of ID has been recently described specifically in 17 female patients with loss of function mutations in *USP9X* (Reijnders et al. 2016). In addition to ID, the syndrome includes congenital anomalies such as choanal atresia, cleft palate, polydactyly, anal atresia, Dandy-Walker malformation as well as a history of thyroid disease, recurrent infections and hearing loss (Reijnders et al. 2016) (Table 1). Here we report two females with ID due to novel mutations in *USP9X*. Both mutations in these children were *de novo* and have not been previously reported. The first individual was a 3 year old female child with intellectual disability and pigmentary abnormalities. She was not overtly dysmorphic and has a relatively milder phenotype than those individuals previously reported, with absence of obvious facial dysmorphisms and other congenital anomalies (Table 1) (Reijnders et al. 2016). However, the pigmentation abnormalities along the lines of Blaschko and hydronephrosis observed in this patient were also observed in the large reported cohort (Reijnders et al. 2016). Of note, her mutation comprises a deletion of the 5'UTR of the *USP9X* gene and, as such, is not clear if this is a complete loss of function mutation. The second individual was more typically dysmorphic, and thus more similar to previously reported individuals (Figure 3). Facial features in individual 2 that have also been described in other females with *USP9X* mutations include a broad forehead,

flat nasal bridge, wide inner canthus, short palpebral fissures, flared nares, a long philtrum and low set posteriorly rotated ears. In addition, individual 2 also presented with post-axial polydactyly, ventriculomegaly, and recurrent respiratory tract infections, similar to previously reported females with loss of function mutations. The clinical findings in these two new cases highlights that mutation of *USP9X* can be associated with a broader phenotypic spectrum in females than previously described.

The female patients reported by Reijnders *et al.* that defined this new intellectual disability syndrome all had *USP9X* loss of function due to stop gain, frameshift and splice-site mutations, in addition to intragenic deletion and whole gene deletions. There was one missense mutation identified that was present in the catalytic domain that was predicted to be loss of function (Reijnders *et al.* 2016). Individual 1 presented here has an interrupted *USP9X* gene due to pericentric inversion resulting in deletion of the 5'UTR, and individual 2 has a frameshift mutation, (*USP9X*): c.4104_4105del (p.(Arg1368Serfs*2)) predicted to be loss of function. Males have been reported with maternally inherited missense mutations in *USP9X* (Homan *et al.* 2014). One male has been reported with a frameshift mutation in the last exon of *USP9X*, expected to escape nonsense mediated decay (Homan *et al.* 2014). However, no males have been reported with proven hemizygous loss-of-function mutation in *USP9X*, suggesting that loss of function in males is likely lethal, which is supported by male, and female, lethality in mice with of conditional loss-of-function of *Usp9x* *-/-* or *Usp9x* *-/y* in the mouse brain (Stegeman *et al.* 2013). The heterozygous carrier females of the conditional *Usp9x* *+/-* deletion in brain were normal at birth and lived into adulthood (Stegeman *et al.* 2013). A milder phenotype was observed in female mice with *Usp9x* *-/-* deletions expressed in the telencephalon structures-only; these mice also lived into adult life but have brain malformations such as small hippocampi and

corpus callosum (Stegeman et al. 2013). Interestingly, the reported male patients with missense mutations in this gene (Homan et al. 2014; Paemka et al. 2015) do not exhibit any of the congenital malformations described in females with loss of function mutations, although they do have intellectual disability, short stature and occasionally epilepsy (Paemka et al. 2015). Furthermore, these male patients typically inherit their missense mutation mutations from carrier mothers with no apparent features of disease (Homan et al. 2014). This dichotomy in mutation type and inheritance pattern is similar to that seen observed in individuals and families with *DDX3X* mutations.

De novo heterozygous mutations in *DDX3X* in females cause a form of intellectual disability characterized by an associated movement disorder, seizures and central nervous system abnormalities. The *de novo* mutations identified in females, which include stop, frameshift and missense changes, are all predicted to be loss of function based on zebrafish studies (Snijders Blok et al. 2015). However, like *USP9X*, missense mutations in *DDX3X* have also been identified in males as a cause of intellectual disability (Snijders Blok et al. 2015). In these families, carrier females of the missense mutations were not affected with ID, and further, missense *DDX3X* mutations found in males have failed to demonstrate loss of function in the zebrafish model (Snijders Blok et al. 2015). The mechanisms of action of these milder missense mutations is still unclear but is speculated to be related to the dosage sensitivity of the *DDX3X* gene.

In individual 1, it is not clear if the 5'UTR deletion of *USP9X* truly causes loss of function of the gene. Deletions of the 5'UTR may not necessarily lead to complete loss of function, although this may lead to reduced amount of protein due to impact on the gene promoter and regulatory elements. It is possible that the deletion of the *USP9X* 5'UTR is such

that some functional gene product can still be produced from this allele. Unfortunately, no cell lines are available to examine protein expression of *USP9X* in individual 1. Residual *USP9X* protein expression from the affected allele may be the contributing factor to this patient's milder presentation, particularly if *USP9X* is dosage sensitive as consistent with mouse experiments (Stegeman et al. 2013) and as *DDX3X* is suggested to be (Snijders Blok et al. 2015).

The milder phenotype in individual 1 could also be due, in part, to favourable X-inactivation, where the unaffected X chromosome is preferentially expressed. *DDX3X* and *USP9X* are located in close proximity to one another at Xp11 and are both known to escape X-inactivation (Zhang et al. 2013). In some genes such as *DDX3X* this escape is not fully complete and is considered a partial X-inactivation (Reijnders et al. 2016). The presence of pigmentary changes along the lines of Blaschko, in some females, does suggest a degree of lyonization (Happle 2006; Reijnders et al. 2016). In other conditions where the relevant gene is known to escape X-inactivation, either partially or more completely, skewing may not fully rescue the disease phenotype if normal development requires biallelic expression. While not significant, preferential X-inactivation (80:20) was seen in the "mild" first individual with the hyper-pigmentation along the lines of Blaschko. In addition, three of five previously reported females with heterozygous loss-of-function mutations in *USP9X* tested for skewing were also found to be greater than 90% skewed (Reijnders et al. 2016). Skewing in blood lymphocytes does not so far appear to be correlated with the severity of symptoms, but this may not represent the relevant developmental stage or tissue specific effects of X-inactivation and skewing (Reijnders et al. 2016). It is possible that the relative extent of the escape from X-inactivation of the affected versus unaffected allele, and the extent of subsequent X-inactivation in the relevant tissues of the central nervous system, may be contributing to the clinical spectrum in affected females.

In conclusion, we have reported a female with a *de novo* pericentric inversion that is associated with a 0.326 Mb deletion in the 5' UTR of *USP9X*, and another female with a *de novo* frameshift mutation. In contrast with the second individual, the first individual presented more mildly than other females described in the literature with *USP9X*-related ID. While she had some differences that were in keeping with other *USP9X* mutation affected females, such as hydronephrosis and pigmentation abnormalities, her presentation was milder with the relative absence of major congenital malformations, and we suggest that mutations in *USP9X* can have a wider spectrum of presentation in females than previously reported. Further identification and study of male and female patients with varying types of *USP9X* mutations may improve our understanding of genotype-phenotype correlation, dosage and gender specific variability associated with this rare ID syndrome.

Conflicts of Interest

The authors have no conflicts of interests to declare.

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Figure legends

Figure 1. Title: Deletion of 5' USP9X. Red arrows demarcate the inversion breakpoints. The red horizontal bar represents the deletion. Blue rectangles represent exons of RefSeq genes within the region and the images was modified from UCSC Genome Browser, GRCh37/hg19 assembly (<https://genome.ucsc.edu/index.html>).

Figure 2. Title: Images of brain, hands and feet of Individual 1. A- Axial single shot fast spin echo (SSFSE) of Case 1. Shows prominence of extra-axial spaces and lateral ventricles with otherwise normal structure and sulcal gyri. B-Coronal SSFSE views of Individual 1 showing normal structure and gyri of cortex and cerebellum. C, D shows hands and feet of case 1. Digits, nails and creases were described as normal though with hypermobility to small joints of hand and slender feet.

Figure 3. Title: Images of Individual 2; A,C-D show broad forehead, prominent hemangioma, short palpebral fissures with a wide inner canthus. The nose has a flat nasal root, and a wide base with flared nares. Philtrum is long. B-shows the low set, rotated helix with attached lobule. C-the image (arrow) shows the post-axial polydactyly (tied off with string).

Table 1. Clinical features in females with USP9X mutations*

Features	Individual 1	Individual 2	Rejinders et al., 2016
Intellectual disability	Mild-moderate	Moderate	17/17
Prominent forehead	No	Yes	n/a
Low nasal bridge, prominent nose with flared alae n/asi and a broad n/asal base	No	Yes	n/a
Thin upper lip	Yes	Yes	n/a
Low set ears and posteriorly rotated ears	No	Yes	n/a
Attached lobules	Yes	Yes	n/a
Short stature	No	No	9/17
Eye abnormalities	No	Myopia	10/17
Choanal atresia	No	No	6/17
Cleft palate/high arched palate/bifid uvula	No	Yes	5/17
Dental abnormalities	Caries	Dental Crowding	12/17
Asymmetric hypomastia	No	Yes	5/17
Heart defect	No	Cardiomyopathy	7/16
Urogenital abnormality	Hydronephrosis	None	5/17
Sacral dimple	No	No	5/17
Hip dysplasia	No	No	8/17

Post-axial polydactyly	No	Yes	9/17
Abdominal wall abnormality	No	No	2/17
Anal atresia	No	No	9/17
Seizures	No	Yes	4/17
Hypotonia	Mild hypotonia with hypermobility	Yes	8/17
Dandy Walker malformation	No	No	5/13
Corpus callosum abnormalities	No	No	8/13
Cerebellar hypoplasia	No	No	6/11
Ventriculomegaly	No	Yes	8/11
Thin brain stem	No	No	3/10
Abnormal gyration pattern	No	No	5/10
Hearing loss	No	Yes	11/17
Pigmentary abnormality	Lines of Blaschko present to torso	No	11/17
Hypertrichosis	No	No	5/17
Limb length discrepancy	No	No	7/17
Malignancy	No	No	2/17
Recurrent infections	No	Yes	9/17
Thyroid abnormality	TSH and free T4 in normal range	TSH normal	6/17

*Modified from Rejinders et al., 2016 [7]

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