Two Novel Pathogenic *MID1* Variants and Genotype-Phenotype Correlation Reanalysis in X-Linked Opitz G/BBB Syndrome

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Established Facts

- To date, 103 index male X-linked Opitz G/BBB syndrome (XLOS) patients with a *MID1* pathogenic variant have been identified.
- The major clinical features of XLOS seem to be associated with specific impaired *MID1* domains.

Novel Insights

- Two novel pathogenic *MID1* variants were characterized in 4 male XLOS patients.
- No genotype-phenotype correlation could be established between clinical features and the type of *MID1* mutations.
- More accurate phenotyping of XLOS patients may disclose potential genotype-phenotype correlations in the future.

Keywords

Genotype-phenotype correlation · *MID1* pathogenic variants · X-linked Opitz G/BBB syndrome

Abstract

X-linked Opitz G/BBB syndrome (XLOS) is a multisystemic congenital condition, caused by mutations in the midline-1 gene (*MID1*), characterized by a large inter- and intrafamilial phenotypic variability and often associated with intellectual

disability (ID). We report clinical, genetic, and molecular findings in 4 patients with typical XLOS dysmorphic features belonging to 2 unrelated families. Two novel pathogenic loss-of-function *MID1* variants, a maternally inherited c.1656del and a de novo c.1215_1228dup, were identified. Subsequently, we performed a genotype-phenotype analysis using data from 91 male XLOS patients. To test the mutation impact on the phenotype; the type of mutation, the *MID1*-impaired domain and function were compared with the presence of each of the major clinical features (hyper-

telorism, clefts of the lip and/or palate, laryngo-tracheo-esophageal abnormalities, hypospadias and ID) and minor clinical features (brain, heart, and anal defects). No statistically significant correlation was found with these features. Further investigations, as well as exhaustive and unequivocal phenotyping, may be required to improve our knowledge of the biological mechanisms underlying this syndrome and to provide more adequate disease management.

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Opitz G/BBB syndrome is a multiple midline congenital condition recognizable by minor facial anomalies such as telecanthus/hypertelorism, a broad nasal bridge, and clefts of the lip and/or palate (CLP). Other midline features include congenital heart defect, imperforate anus, hypospadias, malformations of the central nervous system (including hypoplasia of the corpus callosum and agenesis of cerebellar vermis), laryngo-tracheo-esophageal (LTE) defects, and intellectual disability/developmental delay (ID/DD) [Fontanella et al., 2008]. The disorder can be inherited in an X-linked (OMIM 300000) or autosomal dominant (OMIM 145410) manner [Robin et al., 1995; Kruszka et al., 2015]. The X-linked form, X-linked Opitz G/BBB syndrome (XLOS), is caused by pathogenic variants in the midline-1 gene (*MID1* – OMIM *300552) located in the short arm of the X chromosome (Xp22.2). *MID1* spans 400 kb and is composed of 10 exons which encode a 667-amino acid protein belonging to the tripartite motif subfamily of zinc finger proteins [Quaderi et al., 1997]. XLOS shows variable penetrance particularly in female carriers who can be mildly affected, presenting with hypertelorism or, more rarely, other clinical features such as short uvula or lack of an incisor, and CLP. The extensive inter- and intrafamilial phenotypic variability is yet to be explained [Preiksaitiene et al., 2015].

To date, 90 different pathogenic variants in *MID1* have been described, including missense and nonsense mutations, small insertions and deletions, splice site alterations, exon deletions and duplications as well as deletions of the complete gene [Quaderi et al., 1997; Gaudenz et al., 1998; Schweiger et al., 1999; Cox et al., 2000; De Falco et al., 2003; Winter et al., 2003; Pinson et al., 2004; So et al., 2005; Cho et al., 2006; Mnayer et al., 2006; Shaw et al., 2006; Ferrentino et al., 2007; Fontanella et al., 2008; Hsieh et al., 2008; Taylor and Aftimos, 2010; Ruiter et al., 2010; Zhang et al., 2011; Hu et al., 2012; Huning et al., 2013; Migliore et al., 2013; Ji et al., 2014; Preiksaitiene et al., 2015]. *MID1* consists of the RING finger (RING), zinc-binding B-box-1

(B1) and B-box-2 (B2) domains, followed by a coiled-coil region (CC) and C-terminal subgroup one signature (COS), fibronectin type III repeat (FN3) and PRY-SPRY domains [Quaderi et al., 1997; Du et al., 2014]. Functionally impaired *MID1* variants have a lower affinity for microtubules, causing degradation of protein phosphatase 2A (PP2A), a microtubule-associated protein. Controlling PP2A is essential for proper hippocampus development, and PP2A absence seems to have a direct impact on the core-midline phenotype observed in XLOS patients [Troockenbacher et al., 2001; Schweiger and Schneider, 2003]. Furthermore, a protein complex including *MID1* is involved in mRNA transport and translation by interacting with several proteins, such as elongation factor I alpha, RACK1, Annexin A2, and proteins of the small ribosomal subunits [Aranda-Orgillés et al., 2008, 2011]. Some authors found associations between impaired *MID1* B-box domains and frequent anomalies, or between variants in the FN3 domain and milder phenotypes. However, these results were not confirmed in other recent studies where more patients were included [Li et al., 2015].

We performed a genotype-phenotype analysis by using data from 91 XLOS index patients, aiming to investigate the prevalence of the associated clinical features to assist in establishing a proper prognosis and disease management, and ultimately increase our understanding of the mechanisms of disease.

Case Report

In family A, patients presented with hypertelorism, telecanthus, and anteverted nostrils. The proband, A-III:3, also had CLP, hypospadias, and swallowing difficulties, necessitating a tracheotomy which was removed at 4 years of age. Furthermore, the boy also showed mild early global DD with no learning difficulties and a normal IQ value at the age of 7 years. His maternal cousins, A-III:1 and A-III:2, showed low-set and dysplastic ears besides hypertelorism, telecanthus, and anteverted nostrils. The cousins had a normal IQ and A-III:1 also had hypospadias. Both mothers had hypertelorism. In family B, the proband, B-II:1, presented with hypertelorism, telecanthus, anteverted nostrils, abnormal ears, inverted nipples, and hypospadias. This boy's psychomotor development and IQ were normal (Fig. 1; Table 1). His mother (I:2) showed normal facial features with no hypertelorism.

Methods and Results

In 4 XLOS male individuals belonging to 2 unrelated families, 2 novel *MID1* variants were characterized by Sanger sequencing analysis of all exons and respective flanking regions of the gene (online suppl. Fig. 1; see www.

Fig. 1. Craniofacial abnormalities of the XLOS male patients with *MID1* pathogenic variants. The 3 patients of family A have hyper-telorism, telecantus, and anteverted nostrils. Proband A-III:3 (19 months) shows additional cleft of the lip and palate, and tracheotomy. His maternal cousins (A-III:1, 21 years old and A-III:2, 16

years old) also present with low-set and dysplastic ears. The proband of family B, B-II:1 (left picture: 6 months old; right picture: 3 years old), has hypertelorism, telecantus, anteverted nostrils, and abnormal ears.

karger.com/doi/10.1159/000479177 for all online suppl. material). PCR products were purified using the Illustra™ ExoStar™ 1-Step, (GE Healthcare Life Sciences®, Little Chalfont, UK) and sequenced using the BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems™, Foster City, CA, USA). Exonic *MID1* deletions and duplications were excluded by MLPA analysis (SALSA MLPA P233 *MID1* probe mix, MRC-Holland, Amsterdam, The Netherlands). In family A, a frameshift pathogenic variant, c.1656del, p.(Trp552Cysfs*35)/r.(spl?), was identified. This variant occurs at the exon 10 acceptor exonic splice site and presumably affects splicing (GeneSplicer 12.13, MaxEnt

12.38, HSF 94.37). The proband's maternal cousins, A-III:1 and A-III:2, are hemizygous for the same *MID1* pathogenic variant. His mother (II:6) and maternal aunt (II:1) are both heterozygous for the pathogenic variant. Regarding the deceased brothers, one was a stillbirth, and the other brother died at 19 months due to an unclassified "disorder." In both cases, the presence of major anomalies was not mentioned. Both maternal grandfather and grandmother had several brothers and sisters with healthy offspring.

In family B, a frameshift *MID1* variant was identified in patient B-II:1: c.1215_1228dup, p.(Ser410Thrfs*34).

Table 1. Clinical features observed in the male XLOS patients with *MID1* pathogenic variants

	Family A			Family B
	III:3 (proband)	III:1 (cousin)	III:2 (cousin)	II:1 (proband)
Hypertelorism/telecantus	+/+	+/+	+/+	+/+
Clefts of the lip/palate	+/+	-/-	-/-	-/-
Dysplastic external ears	-	+	+	+
Anteverted nostrils	+	+	+	+
Laryngo-tracheo-esophageal defects	+	-	+	+
Hypospadias	+	+	-	+
Anal defects	-	imperforate and ectopic anus	ectopic anus	-
Heart defects	hypertrophic cardiomyopathy	-	-	-
Brain defects	-	-	-	-
Intellectual disability	-	-	-	-
Additional findings	-	inguinal and umbilical hernias clinodactyly of 5th fingers sindactyly of 2nd and 3rd toes	chronic constipation	supranumerary teeth inverted nipples mos 45,XY-21[3]/46,XY[50]

XLOS, X-linked Opitz G/BBB syndrome; -, absent; +, present.

This genetic alteration was not present in his mother's (I:2) blood or saliva DNA samples (saliva was collected by Oragene[®] DNA Self-Collection kit, DNA Genotek, Ottawa, ON, Canada). To confirm the correct identification of the samples and their familial relationship, 28 short tandem repeats, *AMELX* and *ZFX* genes (Devys Complete v2 QF-PCR kit, Devyser AB, Hägersten, Sweden) were used (online suppl. Fig. 1). Additionally, in patient B-II:1, karyotype analysis of peripheral blood lymphocytes identified a low-level monosomy of chromosome 21 mosaicism: mos 45,XY,-21[3]/46,XY[50]. No other tissues were available to further assess the mosaicism level. As the clinical descriptions of patients with a mosaic monosomy 21 [Toral-Lopez et al., 2012] are clearly distinct from our patient's clinical outcome and the mosaic mutation occurs only in a low percentage of white blood cells, the contribution of the chromosome 21 monosomy to the phenotype was considered to be minor. Assessment of the X-chromosome inactivation pattern was done by using a Humara assay [Allen et al., 1992] in the peripheral blood DNA of the patient's mother (I:2; online suppl. Fig. 1). In accordance with previously published criteria, X-chromosome inactivation ratios of less than or equal to 80:20 were considered "random," and ratios greater than 90:10 were considered "highly skewed" [Amos-Landgraf et al., 2006]. No significant skewing (>80:20) was detected in any of the 3 samples analyzed.

All variants were described using reference sequence NM_000381.3 following the HGVS recommendations (<http://www.hgvs.org/mutnomen/>) [den Dunnen and Antonarakis, 2001] and validated using the Mutalyzer (<https://mutalyzer.nl/>). *MID1* transcript information was obtained at Ensembl (http://www.ensembl.org/Homo_sapiens/Transcript/). *MID1* conserved domains were represented according to NCBI Conserved Domain database (<http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>). 1000 Genomes Project, ExAC Browser, ClinVar, and dbSNP databases were used to establish the allele frequency of novel variants. Further prediction of clinical pathogenicity was done by GeneSplicer Web Interface (http://www.cbcb.umd.edu/software/GeneSplicer/gene_spl.shtml; GeneSplicer threshold ≥ 0), MaxEntScan (http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html; MaxEnt threshold ≥ 0), and Human Splicing Finder (<http://www.umd.be/HSF3/>; HSF threshold ≥ 65) to score putative splice sites, and Combined Annotation Dependent Depletion scoring (<http://cadd.gs.washington.edu/score>; CADD threshold ≥ 15) to score the deleterious effects of single nucleotide variants [Kircher et al., 2014].

Genotype-Phenotype Correlations

A clear genotype-phenotype correlation would assist in establishing a diagnosis and prognosis as well as disease

management options. Therefore, we performed a review of the previously published 103 index male XLOS patients and described 2 in this study (Fig. 2; online suppl. Table 1). One hundred and five index cases out of 139 XLOS patients were selected for analysis to ensure independent observations. Fourteen cases were excluded from further analyses (for details see online suppl. information). Therefore, only 91 XLOS index patients (80 different variants) were considered. In this subgroup of patients, only 22 had information regarding the complete phenotype. The remaining patients included cases with absent information on: structural brain ($n = 68$), and anal defects ($n = 58$), ID/DD ($n = 52$), heart defects ($n = 49$), LTE defects ($n = 37$), CLP ($n = 29$), hypospadias ($n = 17$), and hypertelorism ($n = 13$). To test the mutation impact on the phenotype, several variables were compared with the presence of each of the major and minor clinical features. The variables tested included the type of mutation, protein domains affected by the mutation, presence of a loss-of-function mutation changing transcript size, and mutations that disrupt protein regions with a known biological role. Association between variables was performed using Pearson χ^2 test and Fisher exact test, SPSS for Windows, version 23 (IBM-SPSS, Chicago, IL, USA). A p value of <0.05 was considered statistically significant. Correction for multiple testing was performed using the Bonferroni test [Bonferroni, 1935].

The types of *MID1* pathogenic variants include premature stop codon (44/92; 47.8%), followed by amino acid changes (23/92; 25.0%), and putatively splicing defects (13/92; 14.1%). XLOS major clinical features (present in $\geq 50\%$ of the patients) are hypertelorism (89/90;

Fig. 2. Alignment of XLOS patients according to the mutation position along the *MID1* functional domains and respective clinical features. *MID1* protein domains and gene are represented by rolls and rectangles respectively (not at scale). Each vertical line corresponds to a different index case (see main text for references), and probands described herein are highlighted with a star. Missense mutations are distinguished from the loss of function by vertical black lines. Each clinical feature is symbolized by a colored rectangle, otherwise indicates absence of that feature. Unrelated index cases with the same mutation are connected above, and those with a different mutation affecting the same amino acid are connected below. (a) c.1-?_2004+?del 1-?; (b) c.-400_660+?del/c.-330_660+?del; (c) c.-56-?_660+?del; (d) c.1-?_660+?del; (e) c.1-?_660+?dup; (f) c.661-?_756+?del; (g) arr(Xp22.2)(10,466,103–10,523,151)×2; (h) c.1142_1285del – complex rearrangement of exon 6. B1, zinc-binding B-box1; B2, zinc-binding B-box2; CC, a-helical coiled-coil domain; COS, C-terminal subgroup one signature; FN3, fibronectin type III repeat; Rel. Freq, relative frequency (number of index cases with a given clinical feature among those with available data); RING, ring finger motif; PRY, SPRY-associated domain; SPRY, domain in SPLa and the RYanodine receptor.

98.9%), hypospadias (79/86; 91.9%), LTE defects (57/66; 86.4%), CLP (51/74; 68.9%), and ID/DD (29/51; 56.9%). Minor clinical characteristics present in <50% of the patients include structural anal (20/45; 44.4%), brain (15/35; 42.9%), and heart defects (21/54; 38.9%).

We did not observe an association between the presence of any of the clinical features assessed and the affected *MID1* domains: RING, B1, B2, CC, COS, FN3, PRY, and SPRY (hypertelorism $p = 1.451$, hypospadias $p = 1.462$, LTE defects $p = 1.558$, CLP $p = 1.513$, ID/DD $p = 1.642$, structural anal $p = 0.609$, heart $p = 0.256$, and brain defects $p = 0.256$), nor frequencies of any mutation type (hypertelorism $p = 1.451$, hypospadias $p = 1.462$, LTE defects $p = 1.558$, CLP $p = 1.513$, ID/DD $p = 1.642$, structural anal $p = 0.176$, heart $p = 0.354$, and brain defects $p = 0.193$). There was also no association between the frequency of loss-of-function mutations affecting all transcripts and those affecting only longer transcripts in patients with any of the XLOS clinical features (hypertelorism $p = 1.624$, hypospadias $p = 1.642$, LTE defects $p = 1.757$, CLP $p = 1.730$, ID/DD $p = 1.905$, structural anal $p = 0.445$, heart $p = 0.880$, and brain defects $p = 0.653$). Finally, we addressed a potential genotype-phenotype association between the 2 *MID1* regions with a well-characterized biological role, ubiquitination and microtubules interaction, and XLOS clinical features (hypertelorism $p = 1.451$, hypospadias $p = 1.462$, LTE defects $p = 1.558$, CLP $p = 0.661$, ID/DD $p = 0.609$, structural anal $p = 0.741$, brain $p = 0.698$, and heart defects $p = 0.032$). The observed association between heart defects and *MID1* function ($p = 0.032$) was classified as not significant after correction for multiple testing (Bonferroni-corrected $p = 0.128$). Our findings expand the clinical and molecular spectrum of *MID1* and do not show any significant genotype-phenotype correlation.

Discussion

Two novel frameshift pathogenic variants, c.1656del and c.1215_1228dup, presumably resulting in a premature stop codon p.(Trp552Cysfs*35)/r.(spl?) and p.(Ser410Thrfs*34), respectively, were identified in 4 patients. The X-chromosome inactivation patterns in the blood of unaffected carrier females were not skewed, which is in concordance with previously published data [Pinson et al., 2004] and may explain the hypertelorism in carrier mothers (family A: II:1 and II:6). None of the 4 male XLOS patients have a neurodevelopmental or intellectual disability.

Although XLOS is defined as an ID syndrome, this complies with previously reported patients without ID.

Our analyses suggest that there is no correlation between specific major or minor clinical signs and any of the tested variables. This confirms the data of Li et al. [2015]. However, some data due to incomplete phenotyping or relatively poorly described clinical details of patients with XLOS are missing (i.e., full IQ assessment is often missing). Fontanella et al. [2008] suggested an association between truncating mutations (frameshift and nonsense) and the presence of assessed brain deformities (11 out of 12 cases). In the same study, a possible association between CLP abnormalities and mutations affecting splicing of *MID1* transcripts was observed. These results could not be confirmed in our study.

Further studies involving more detailed phenotyping in a larger cohort, integrating the data from familial cases, are required to explore the role of specific pathogenic variants in the genesis of this disorder. Therefore, we would argue for a careful assessment of major and minor clinical features of XLOS patients for a better understanding of the molecular role of the protein in the disease phenotype, which may have important clinical and therapeutic implications.

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Statement of Ethics

Participants, or their legal representatives, signed informed consent for the use of DNA samples in research and biobanking as well as permission to publish all images. This study has been approved by the medical ethical committee of the Centro Hospitalar do Porto, EPE – REF 2014.203 (144-DEFI/173-CES). The work was supported by national funds: FCT Strategic Project UID/Multi/00215/2013 and Centro Hospitalar do Porto, EPE research grants Bolsa de Investigação DEFI 2014 (I.M.). All authors have read the journal's authorship agreement and the manuscript has been reviewed and approved by all authors.

Disclosure Statement

The authors have no conflicts of interest to declare.

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