Successful pregnancies in an adult with Meier-Gorlin syndrome harbouring biallelic *CDT1* variants

Running title: Successful pregnancies in MGORS

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Abstract

Meier-Gorlin syndrome is an autosomal recessively inherited disorder of growth retardation, accompanied by microtia and patellae a/hypoplasia and characteristic facies. Pathogenic variants in genes associated with the initiation of DNA replication underlie the condition, with biallelic variants in *CDT1* the most common cause. Using 10X Chromium genome sequencing, we report *CDT1* variants in an adult female, with an inframe amino acid deletion inherited *in trans* with a deep intronic variant which likely serves as the branchpoint site in intron 8. Splicing defects arising from this variant were confirmed through *in vitro* analysis.

At 49 years, she represents the oldest patient with a molecular diagnosis described in the literature and is the first reported patient with Meier-Gorlin syndrome to have carried a successful pregnancy to term. Both of her pregnancies were complicated by postpartum haemorrhage and upon subsequent necessary hysterectomy, revealed uterine abnormalities. There is scant knowledge on reproductive ability and success in patients with Meier-Gorlin syndrome. Successful pregnancies amongst other clinically recognisable forms of primordial dwarfism have also not been described previously. This case is therefore of clinical interest for many forms of inherited growth retardation, and will assist in providing more information and clinical guidance for females of reproductive age.

Keywords: Meier-Gorlin syndrome, growth disorders, pregnancy, DNA replication

Introduction

Meier-Gorlin syndrome (MGORS, MIM: 224690) is a rare form of primordial dwarfism. The cardinal features are reduced growth, which is often proportionate (reduced stature and head size), with microtia and patella a/hypoplasia(Bongers et al., 2001). There are facial characteristics of microstomia with full lips, retro/micrognathia and a prominent nose, which becomes more noticeable with age. Other common features include congenital emphysema and external urogenital abnormalities of labial hypoplasia in females and cryptorchidism in males(de Munnik et al., 2015). In females, breast development is almost universally absent(de Munnik et al., 2015).

MGORS is a disorder of DNA replication initiation, with nine disease-associated genes identified to date(Bicknell, Bongers, et al., 2011; Bicknell, Walker, et al., 2011; Burrage et al., 2015; Fenwick et al., 2016; Guernsey et al., 2011; Knapp et al., 2020; Vetro et al., 2017). DNA replication is initiated in late mitosis and G1 phases of the cell cycle, where the Origin Recognition Complex (ORC subunits 1-6) bind to genomic origins in chromatin. CDC6 and CDT1 coordinate the recruitment of the inactive MCM helicase to these sites, to form the pre-replication complex (preRC). Additional proteins, as part of the pre-initiation complex (preIC), bind to the preRC at the onset of S phase, to unwind the DNA and allow access for the DNA polymerases, commencing DNA replication(Fragkos et al., 2015). All the proteins encoded by MGORS-associated genes serve essential roles in licensing of genomic origins as part of the pre-replication complex (ORC1, ORC4, ORC6, CDT1, CDC6, MCM5), as a negative regulator of DNA replication (GMNN), or a component of the replication pre-initiation complex (CDC45) or the replisome (DONSON)(Bicknell, Bongers, et al., 2011; Bicknell, Walker, et al., 2011; Burrage et al., 2015; Fenwick et al., 2016; Guernsey et al., 2011; Karaca et al., 2019; Knapp et al., 2020; Vetro et al., 2017). CDT1 acts to assist the

loading of the two heterohexameric MCM helicase complexes onto the ORC complex, and associates with the ORC complex in a transient manner(Ticau et al., 2015).

Biallelic variants in *CDT1* represent the most common cause of MGORS(Bicknell, Bongers, et al., 2011; de Munnik, Bicknell, et al., 2012). Almost all pathogenic variants are inherited in a compound heterozygous manner and in most cases there is a truncating variant on one allele. The studied missense variants generally load MCM onto ORC-bound genomic origins less efficiently, through impairing the ability of CDT1 to interact with the MCM helicase(Pozo et al., 2018).

Here we describe an adult female with MGORS, in whom we confirm biallelic *CDT1* likely pathogenic variants. This individual has been able to successfully carry two pregnancies to term despite severe complications, and to our knowledge is the first reported individual with MGORS to do so. This knowledge will be useful for clinical advice and monitoring of other patients with MGORS, particularly females of reproductive age, as well as being of clinical interest for other forms of primordial dwarfism syndromes.

Methods

Ethical approval for this study was provided by the New Zealand Health and Disability Ethics Committee (16/STH/3) and Scottish Multicentre Research Ethics Committee (05/MRE00/74). Consent (including the use of photos) was obtained from the patient.

DNA was obtained from a blood sample by standard extraction methods. Chromium genome sequencing and bioinformatic processing and filtering of sequence data were undertaken as previously described(Knapp et al., 2020). Variants were confirmed by Sanger sequencing (oligonucleotides listed in Supplementary Table 1) .Variant nomenclature is based on

RefSeq: NM_030928.3. Variant information has been uploaded to the LOVD-CDT1 database.

Minigene splicing assay

CDT1 exons 8–10 was PCR amplified from control genomic DNA using a sense oligonucleotide located within intron 7 (incorporating approx. 90 bp flanking 5′ intronic sequence) and an antisense oligonucleotide located within exon 10 (the last *CDT1* coding exon) fused to an introduced splice donor sequence. Gateway cloning was used to insert the PCR product into the pSpliceExpress vector (Addgene: #32485), which was a kind gift from Stefan Stamm(Kishore et al., 2008). The intronic *CDT1* variant was introduced by site-directed mutagenesis, and all constructs were verified by Sanger sequencing. Oligonucleotides are listed in Supplementary Table 1. HEK293FT cells were transiently transfected with plasmid DNA using Lipofectamine 2000 (Invitrogen). After 24 hours, RNA was extracted and purified using a Qiagen RNeasy Mini Kit (Qiagen), and then cDNA was synthesised using the SuperScript IV VILO Master Mix with ezDNase kit (Invitrogen). cDNA was PCR amplified using plasmid specific primers, PCR products were visualised by gel electrophoresis and gel bands extracted using a GeneJET Gel Extraction kit (Thermo Fisher Scientific) and sequenced.

Results

Clinical History

The patient, P1, is a 49 year old female who had been clinically diagnosed with MGORS. Antenatal history was unremarkable, except for poor growth in the last trimester with a birth weight of 2.50 kg (-1.83 SD) at term. Ears appeared initially overfolded and simple, however as an adult they are a normal shape but very small (4cm in length). P1 was a poor feeder with

failure to thrive and recurrent respiratory infections and diarrhoea, for which coeliac disease was ruled out.

Bilateral absence of patellae was confirmed by X-ray at eight years of age, with subsequent surgical interventions causing limited extension of the right knee. She has small feet with no arch present. Cognitive development has been normal. She has suffered from chronic lung infections as an adult with X-ray evidence of mild emphysema. Puberty occurred at 17 years of age in the absence of any breast development. Menses were short and irregular and following one miscarriage at 8 weeks, she went on to have two successful pregnancies, at ages 21 and 31 yrs. The first pregnancy was complicated by preeclampsia and delivery was by caesarean-section. The baby was healthy, weighing 2.07kg. The second pregnancy followed several years of infertility and also required a caesarean-section as the birth canal was described as too small. The baby was healthy, weighing 2.78kg. This second labour was complicated by severe haemorrhage requiring a hysterectomy after the second birth. Her uterus was noted as appearing “aged” and was unusually thin and friable.

On recent examination her head circumference was 53 cm (-1.81 SD), height 150.1cm (-2.23 SD) and weight of 57.4 kg (-0.02 SD). She has a young appearance with a normal facial appearance apart from the smaller ears (Fig. 1). Hand size is within normal range (17.3 cm with a middle finger length of 7.5cm) and is not proportionate to her stature. She has hyperextensibility of several joints and suffers from dental crowding with irregular spacing of teeth.

Molecular Analysis

Chromium genome sequencing uses linked-read barcode technology to achieve separation of chromosomes prior to sequencing. This protocol permits phasing of alleles and is of

particular utility in studying disorders inherited in an autosomal recessive fashion(Knapp et al., 2019) and in cases where no parental DNA is available. Following processing of the linked-read genome sequencing raw data using LongRanger, we first searched for biallelic variants in genes previously established to be associated with MGORS. Through this variant analysis we identified P1 was heterozygous for an inframe deletion, c.1078_1080del, p.(Ala360del), in *CDT1*, the most commonly associated MGORS gene. Only one individual has been reported as heterozygous for this variant in gnomAD and Ala360 shows strong conservation through to *D. melanogaster*. According to ACMG/AMP guidelines(Richards et al., 2015), this variant can be classified as likely pathogenic (PM2, PM4, PP3, PP4). Given the strong candidacy of this variant, we therefore considered any alterations inherited *in trans* with this variant. While there were no candidate coding variants identified on the phased second haplotype, there was a deep intronic variant, c.1276-24A>G, present in intron 8 of *CDT1*. *In silico* analysis of this variant (Alamut Visual) suggested the adenine allele might act as the branchpoint ribonucleotide site during splicing of intron 8; a substitution to a guanine ribonucleotide was predicted to significantly impact splicing(Gao et al., 2008). Both variants were confirmed by Sanger sequencing.

To investigate any effects on splicing caused by the intronic variant, we undertook a minigene splicing assay. We constructed the pSpliceExpress plasmid to contain exons 8-10, flanked by a 5′ non-coding genic sequences and an introduced 3' splice donor (Fig 2A). Because exon 10 is the last *CDT1* coding exon an introduced splice donor sequence was necessary to facilitate efficient splicing in the minigene assay. We transfected HEK293FT cells with plasmid harbouring the reference *CDT1* ("Ref") or a plasmid harbouring the intronic variant ("Variant") and undertook RT-PCR analysis on extracted RNA (Fig. 2B), sequencing the resultant RT-PCR products (Fig. 2C). RNA transcribed from the plasmid containing the reference sequence produced a single product (with the exception of a plasmid

exon only product), with correct splicing of intron 8 and canonical exon 8 and 9 boundary sequences. In contrast, RNA from the variant-containing plasmid produced two RT-PCR products. Sequencing of these products confirmed two aberrant splicing products, one in which exon 9 is skipped, which through a frameshift creates a novel C-terminus to the protein (p.Ile426Glyfs*93), which is predicted to be disordered and its stability and functional capacity are unclear. The second product, which is the most predominant, is where intron 8 has not been spliced out of the transcript. This introduces a premature stop (p.Ile426_Ala431delinsValSerArgProGlnTer), and given the location in the transcript, is likely to result in nonsense-mediated decay. Based on these investigations, we are able to classify c.1276-24A>G as a likely pathogenic variant (PS3, PM2, PP3).

Discussion

Here we report the molecular diagnosis of an adult female individual with MGORS using Chromium genome sequencing, where the haplotype phasing assisted in our prioritization of variants segregating *in trans*. We identified biallelic *CDT1* variants and we confirmed deleterious effects of the deep intronic variant by *in vitro* analysis. Pathogenic variants in *CDT1* are the most commonly described in MGORS(Bicknell, Bongers, et al., 2011; de Munnik, Bicknell, et al., 2012). The majority of genotypes are a loss of function variant inherited in a compound heterozygous fashion with a deleterious missense variant; we also observe this, where the intronic variant in our patient likely represents a null allele, as no canonical transcript was detected in the *in vitro* minigene splicing assay. The linked-read phasing sequencing approach was particularly useful in this setting, as branchpoint ribonucleotides are particularly difficult to reliably predict(Gao et al., 2008; Leman et al., 2020).

To our knowledge, this is the oldest patient reported in the literature with a molecular MGORS diagnosis and the first reported who has had multiple successful pregnancies. There is scant knowledge as to the effects of MGORS variants on the reproductive ability of females. In a case series, de Munnik *et al.* noted 2/5 females of appropriate age each had a small uterus with polycystic ovaries, while one female had a shortened uterus with two late miscarriages (with no foetal anomalies detected by autopsy)(de Munnik, Otten, et al., 2012). The two other cases reported both had a normal uterus by transvaginal ultrasound. This case report extends these observations, with our patient successfully carrying two pregnancies to term but with complications requiring a subsequent hysterectomy. Her uterus was noted to be abnormal (thin and with an "aged" appearance); such features may be a longer-term effect beyond those noted in younger patients. While there is obvious variability in the presence and spectrum of abnormal uterine features, cumulatively such abnormalities are becoming a more evident characteristic to be considered in clinical management of females of reproductive age with MGORS.

The underlying pathophysiology of such uterine abnormalities is not immediately clear. There are no mammalian models of MGORS described and due to the obvious complexity of investigating the appearance of a uterus, comparative observations in other genetic disorders are scarce. The cardinal features of microtia and patellar a/hypoplasia suggest that cartilogenesis may be overtly affected by a reduction in DNA pre-replication complex proteins, and other commonly reported features such as mammary hypoplasia, congenital emphysema(Arnaud et al., 2017) and external urogenital abnormalities could fit with this. We speculate that the pathology underlying the uterine abnormalities may be similar in origin.

Successful pregnancies amongst other forms of primordial dwarfism have not been reported, except for one, where the specific type of primordial dwarfism was unclear and the pregnancy complicated by additional factors(Vance et al., 2012). The growth restriction in

Patient P1 is not as severe as other individuals with MGORS or other types of primordial dwarfism, such as MOPD II or Seckel syndrome, which might contribute to better physical support of a pregnancy (the foetus of which is expected to be of normal size), especially in the latter stages. There are likely to be other contributory reasons for the absence of pregnancies in other primordial dwarfism syndromes, such as reduced life expectancy within the reproductive age range, or cognitive impairment. It will be interesting to gain insight from other cases; to learn the diversity and scale of any uterine abnormalities as well as the rates of successful pregnancies across other forms of primordial dwarfism, in order to provide the most appropriate clinical guidance for females of reproductive age with such genetic disorders.

Acknowledgements

We thank the patient and family for their involvement in this research. KMK and LSB are supported by the Marsden Fund, and LSB is supported by a Rutherford Discovery Fellowship, both administered by the Royal Society of New Zealand. I Karen Temple is supported by the NIHR Southampton Biomedical Research Centre, University Hospital Southampton NHS Foundation Trust.

The authors have no conflicts of interest to declare.

Data Availability

Variants have been uploaded to the LOVD-CDT1 database.

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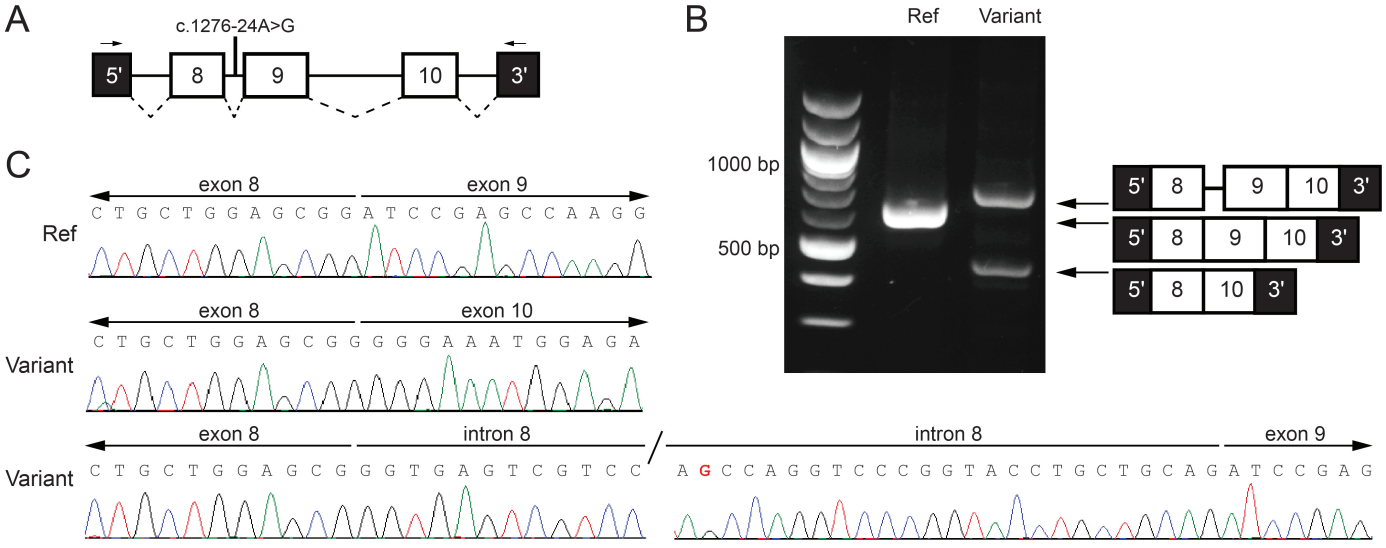
Figure Legends

Figure 1. Clinical features of patient P1. (A, B) P1 has a normal facial appearance, with mild MGORS features including a prominent nose, a full bottom lip, micrognathia and microtia. P1 has a younger appearance relative to her age (49 yrs). (C) PI has relatively proportionate short stature, with relatively shorter lower limbs and spine.

Figure 2. Minigene splicing assay demonstrating no canonical transcript is produced when the variant, c.1276-24A>G, is present. (A) Schematic of the *CDT1* gene included in the mini-gene assay, with the variant position and RT-PCR oligonucleotides indicated. (B) Agarose gel of the RT-PCR products from HEK293FT cells transfected with plasmids containing either the *CDT1* reference sequence ('Ref') or the *CDT1* c.1276–24A>G variant ('Variant'). (C) Sanger sequencing of the RT-PCR products in (B), indicating skipping of exon 9 or retention of intron 8.

Figure 1.

Figure 2



Supplementary Table 1. Oligonucleotides used in this study.

Gateway cloning oligonucleotides	
CDT1_attB1_F	GGGGACAAGTTTCTACAAAAAAGCAGGCTGTGCCTTGAGAGATACCGGG
CDT1_attB1_R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTACCTTGTCCAGCTTGACGTAGG
Mutagenesis oligonucleotides	
CDT1_C1276-24A>G_S	CCTGCTGCCCACTAGCCAGGTCCCGGTACCT
CDT1_C1276-24A>G_AS	AGGTACCGGGACCTGGCTAGTGGGCAGCAGG
RT-PCR oligonucleotides	
Rat_INS2_Ex2_F	CCTGCTCATCCTCTGGGAGC
Rat_INS2_Ex3_R	AGGTCTGAAGGTCACGGGCC
Sequencing oligonucleotides	
CDT1_Ex7_F	CGTAAGCACAGGCCTACCTC
CDT1_Ex7_R	AAGCTCATCACCAAGGCTTC
CDT1_Ex8-9_F	AGAGATACCGGGGACTCCTG
CDT1_Ex8-9_R	GATCAGTGACAGACACCTCTGC
M13_F	GTAAAACGACGGCCAG
M13_R	CAGGAAACAGCTATGAC