Analysis of CDKN1C in fetal growth restriction and pregnancy loss [version 1; peer review: awaiting peer review]

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Abstract

Background: Cyclin-dependent kinase inhibitor 1C (CDKN1C) is a key negative regulator of cell growth encoded by a paternally imprinted/maternally expressed gene in humans. Loss-of-function variants in CDKN1C are associated with an overgrowth condition (Beckwith-Wiedemann Syndrome) whereas “gain-of-function” variants in CDKN1C that increase protein stability cause growth restriction as part of IMAGe syndrome (Intrauterine growth restriction, Metaphyseal dysplasia, Adrenal hypoplasia and Genital anomalies). As two families have been reported with CDKN1C mutations who have fetal growth restriction (FGR)/Silver-Russell syndrome (SRS) without adrenal insufficiency, we investigated whether pathogenic variants in CDKN1C could be associated with isolated growth restriction or recurrent loss of pregnancy.

Methods: Analysis of published literature was undertaken to review the localisation of variants in CDKN1C associated with IMAGe syndrome or fetal growth restriction. CDKN1C expression in different tissues was analysed in available RNA-Seq data (Human Protein Atlas). Targeted sequencing was used to investigate the critical region of CDKN1C for potential pathogenic variants in SRS (n=58), FGR (n=26), DNA from spontaneous loss of pregnancy (n=21) and women with recurrent miscarriages (n=71) (total n=176).

Results: All published single nucleotide variants associated with IMAGe syndrome are located in a highly-conserved “hot-spot” within the PCNA-binding domain of CDKN1C between codons 272-279. Variants associated with familial growth restriction but normal adrenal function currently affect codons 279 and 281. CDKN1C is highly expressed in the placenta compared to adult tissues, which may contribute to the FGR phenotype and supports a role in pregnancy maintenance. In the patient cohorts studied no pathogenic variants were identified in the PCNA-binding domain of CDKN1C.

Conclusion: CDKN1C is a key negative regulator of growth. Variants in a very localised “hot-spot” cause growth restriction, with or without adrenal insufficiency. However, pathogenic variants in this region are not a common cause of isolated fetal growth restriction phenotypes or loss-of-pregnancy/recurrent miscarriages.
Keywords
CDKN1C, intra-uterine growth restriction, fetal growth restriction, Silver-Russell syndrome, IMAGe syndrome, adrenal, placenta, recurrent miscarriage

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Introduction

Cyclin-dependent kinase inhibitor 1C (CDKN1C, also known as P57kip2) (OMIM 600856) is a key negative regulator of cell proliferation that is encoded by a paternally imprinted (maternally expressed) gene on the short arm of chromosome 11 (11p15.4) in humans (Stampone et al., 2018).

Consistent with its role in growth and development, maternally-inherited loss-of-function variants in CDKN1C are found in approximately 5–10% of individuals with the “overgrowth” condition, Beckwith-Wiedemann Syndrome (BWS) (OMIM 130650) (Eggermann et al., 2014). Clinical features of BWS include macrosomia, hyperinsulinism and adrenal tumors.

In contrast, gain-of-function variants in CDKN1C have been shown to cause growth restriction as part of IMAGe syndrome (OMIM 614732) (Arboleda et al., 2012). IMAGe syndrome is characterised by fetal/Intrauterine growth restriction, Metaphyseal dysplasia, Adrenal hypoplasia and Genital anomalies (in males, usually relatively mild hypoplasias and undescended testes) as well as additional features such as hearing loss and hypercalciuria (Bennett et al., 1993; Vilain et al., 1999).

To date, children with IMAGe syndrome have all been found to harbour pathogenic single nucleotide variants (SNVs) in a very specific region of the PCNA-binding domain of CDKN1C (Arboleda et al., 2012; Cabrera-Salcedo et al., 2017). These changes potentially lead to increased activity through increasing protein stability, thereby preventing cell cycle progression into S phase (Borges et al., 2015; Hamajima et al., 2013).

More recently, SNVs in the PCNA-binding domain of CDKN1C have been reported in families with maternally-inherited fetal growth restriction (FGR) without adrenal insufficiency and in familial Silver-Russell syndrome (SRS) (OMIM 180860) (Brioude et al., 2013; Kerns et al., 2014). SRS is characterised by variable clinical features including fetal and post-natal growth restriction, relative macrocephaly, feeding difficulties and characteristic facies. SRS is also described as phenotypically and genotypically opposite to BWS and approximately half of the molecular anomalies are attributed to Chr11p15.5 imprinting clusters, including several individuals with maternal duplication of the locus containing CDKN1C (Bonaldi et al., 2011; Boonen et al., 2016; Schönherr et al., 2007).

These findings suggest that the growth restriction phenotype associated with CDKN1C may be more variable and adrenal insufficiency is not always present.

The aim of this study was therefore to review published CDKN1C variants associated with FGR/IUGR phenotypes, to study CDKN1C expression in different tissues, and to analyse the critical region in CDKN1C in a range of growth restriction and adverse pregnancy phenotypes, with a hypothesis that severe restriction of feto-placental growth may, in some situations, result in pregnancy loss or recurrent miscarriage.

Methods

Review of pathogenic SNVs and population variability

A PubMed search was undertaken (March 2018) using the search terms “CDKN1C”, “human” and “growth”, or “IMAGe syndrome”. Reports focusing on growth restriction phenotypes associated with single nucleotide variants were considered. Population variation in CDKN1C was assessed using the gnomAD browser (http://gnomad.broadinstitute.org; accessed April 2018) (Lek et al., 2016). Protein conservancy analysis was performed using ClustalW in Jalview (Waterhouse et al., 2009).

Analysis of CDKN1C expression

RNA-Seq data for CDKN1C expression was obtained with specific permission from the Human Protein Atlas (Human Protein Atlas available from www.proteinatlas.org) and re-drawn in R (version 3.4.2) (Uhlen et al., 2015).

Study cohorts

The following growth restriction cohorts were included in this study: 1) SRS (n=58) (isolated, non-familial) diagnosed on consensus criteria and where maternal uniparental disomy or H19/IGF2:IG-DMR hypomethylation had been excluded (Wakeling et al., 2017); 2) FGR (n=26) (isolated, non-familial) defined as birth weight less than the 3rd centile, as part of the Baby Bio Bank cohort (UCL-GOS Institute of Child Health & St Mary’s Imperial College London) (Leon et al., 2016). Additional analysis was undertaken in DNA from 3) products of conception (POC) (n=21) where there had been a spontaneous loss of pregnancy and 4) mothers who had a history of recurrent miscarriages (n=71) (at least three miscarriages) where an underlying cause was not known (Baby Bio Bank). An overview of these cohorts is provided in Table 1.

**Table 1. Overview of the cohorts studied.**

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Number</th>
<th>Characteristics</th>
<th>Sequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silver-Russell Syndrome</td>
<td>58</td>
<td>Silver-Russell syndrome; maternal uniparental disomy of chromosome 7 or H19/IGF2:IG-DMR hypomethylation excluded</td>
<td>Sanger</td>
</tr>
<tr>
<td>IUGR/FGR</td>
<td>26</td>
<td>DNA from children with intra-uterine growth restriction (birth weight &lt; 3rd percentile) (Baby Bio Bank)</td>
<td>Next-generation sequencing (mean read depth 37.8)</td>
</tr>
<tr>
<td>Products of conception</td>
<td>21</td>
<td>DNA from lost products of conception between 9–11 weeks gestation</td>
<td>Next-generation sequencing (mean read depth 60.1)</td>
</tr>
<tr>
<td>Recurrent miscarriages</td>
<td>71</td>
<td>DNA from mothers with recurrent miscarriages (&gt;3) and usually a history of live births</td>
<td>Next-generation sequencing (mean read depth 31.5)</td>
</tr>
</tbody>
</table>

Abbreviations: FGR, fetal growth restriction; IUGR, intrauterine growth restriction.
Consent

Ethical Committee approval for the Baby Bio bank was obtained from the Trent Derby Ethics Committee (09/H0405/30) and Ethical Committee approval for the Silver Russell trios was from GOSH Research Ethics Committee (REC No. 1278). Written informed consent was obtained from participants or parents. DNA was extracted from blood lymphocytes, placental tissue or products of conception, as appropriate.

Genomic analysis of CDKN1C by Sanger Sequencing

Direct Sanger Sequencing was undertaken for SRS patients to analyse the PCNA-binding region (codons 213–316) and hotspot (codons 272–281) using primers reported previously (CCDS7738, ENST00000414822.8) (Arboleda et al., 2012). Additional primers pairs were used to sequence the 3’ end of exon 1 and splice site (CDKN1CF: CAGGAGCCTCTCGTGAC; CDKN1CR2: GCTGGAGGGCCACAAAC). Polymerase chain reaction (PCR) was carried out with BIOTALQ DNA Polymerase (BIOLINE, London, UK). PCR products were purified by microclean (Microzone, Haywards Heath, UK) and amplified with BigDyeTerminator v1.1, followed by sequencing on a DNA Analyzer 3070 (Applied Biosystems, California, US). The resulting read-outs were reviewed in Sequencher (v5.3: Gene Codes).

Genomic analysis of CDKN1C by targeted capture array and next generation sequencing

Targeted array capture followed by next-generation sequencing was performed for FGR samples, products of conception and mothers with a history of recurrent miscarriage.

A targeted enrichment custom HaloPlex 501kb–2.5Mb HS panel (Agilent Technologies Inc.) was designed using Agilent SureDesign to capture known and candidate genes for fetal growth disruption, including CDKN1C. This study used designs targeting either 147 (design size 1.391 Mbp) or 257 (design size 2.045 Mbp) genes.

Sequencing libraries were prepared using 50ng of genomic DNA following the manufacturer’s protocol (HaloPlex HS Target Enrichment System for Illumina Sequencing version C1 from December 2016) and as described in principle previously (Guran et al., 2016). This was followed by 2 x 100bp or 2 x 149bp paired end sequencing to a median read depth of 300x on a NextSeq sequencer (Illumina Inc.). The bel files were converted to fastq files using manufacturers recommended guidelines and were then analysed in SureCall software (version 4.0.1.46 (Agilent Technologies) using the HaloPlex Default Method or custom settings (minimum number of read pairs per barcode =1). Samples with a minimum read depth less than 4 at any single nucleotide position were excluded.

Results

Single nucleotide variants in CDKN1C

Review of available data revealed six publications describing isolated individuals (7) or families (4) with IMAGe syndrome and adrenal insufficiency, who had pathogenic variants in a key region of the PCNA-binding domain of CDKN1C affecting codons 272, 274, 276, 278 and 279 (Figure 1, Table 2) (Arboleda et al., 2012; Bodian et al., 2014; Brioude et al., 2013; Hamajima et al., 2013; Kato et al., 2014; Kerns et al., 2014). These codons are highly conserved amongst species (Figure 2).

Multiple individuals from different ancestral backgrounds were found to have p.Asp274Asn or p.Lys278Glu changes.

Variants in CDKN1C associated with familial Silver-Russell syndrome or growth restriction but normal adrenal function were

![Figure 1. Schematic diagram showing the structure of CDKN1C and the clustering of pathogenic variants associated with IMAGe syndrome and/or growth restriction.](image-url)
Table 2. Reported variants in CDKN1C and associated phenotypes.

<table>
<thead>
<tr>
<th>Nucleotide variant</th>
<th>Protein change</th>
<th>Isolated/Familial</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.815T&gt;G</td>
<td>p.Ile272Ser</td>
<td>Familial (3)</td>
<td>IMAGe</td>
<td>Harajima et al., 2013</td>
</tr>
<tr>
<td>c.820G&gt;A</td>
<td>p.Asp274Asn</td>
<td>Isolated</td>
<td>IMAGe</td>
<td>Arboleda et al., 2012</td>
</tr>
<tr>
<td>c.820G&gt;A</td>
<td>p.Asp274Asn</td>
<td>Isolated</td>
<td>IMAGe (Glucocorticoid)</td>
<td>Kato et al., 2014</td>
</tr>
<tr>
<td>c.826T&gt;G</td>
<td>p.Phe276Val</td>
<td>Familial (7)</td>
<td>IMAGe</td>
<td>Arboleda et al., 2012</td>
</tr>
<tr>
<td>c.827T&gt;C</td>
<td>p.Phe276Ser</td>
<td>Isolated</td>
<td>IMAGe</td>
<td>Arboleda et al., 2012</td>
</tr>
<tr>
<td>c.832A&gt;G</td>
<td>p.Lys278Glu</td>
<td>Isolated</td>
<td>IMAGe (Mineralocorticoid)</td>
<td>Bodian et al., 2014</td>
</tr>
<tr>
<td>c.832G&gt;C</td>
<td>p.Lys278Glu</td>
<td>Isolated</td>
<td>IMAGe</td>
<td>Arboleda et al., 2012</td>
</tr>
<tr>
<td>c.836G&gt;C</td>
<td>p.Arg279Pro</td>
<td>Isolated</td>
<td>IMAGe</td>
<td>Bodian et al., 2014</td>
</tr>
<tr>
<td>c.836G&gt;T</td>
<td>p.Arg279Leu</td>
<td>Familial (9)</td>
<td>Silver-Russell</td>
<td>Brioude et al., 2013</td>
</tr>
<tr>
<td>c.842G&gt;T</td>
<td>p.Arg281lle</td>
<td>Familial (15)</td>
<td>IUGR, short stature, IGT/DM</td>
<td>Kerns et al., 2014</td>
</tr>
</tbody>
</table>

Abbreviations: DM, diabetes mellitus; IGT, impaired glucose tolerance; IMAGe, intrauterine growth restriction, metaphyseal dysplasia, adrenal hypoplasia congenita, genital anomalies; IUGR, intrauterine growth restriction

Figure 2. Amino-acid conservancy in the “hot-spot” region of CDKN1C. Red arrowheads represent codons that are mutated in IMAGe syndrome, FGR/IUGR or Silver-Russell syndrome. Yellow asterisks represent complete conservation amongst the species shown.
found towards the carboxyl-terminal region of this “hot-spot" domain (p.Arg279Leu, p.Arg281Ile) (Figure 1).

Analysis of population data from the gnomAD browser showed a complete absence of variants in the key codons listed. Very rare heterozygous SNVs were found that are predicted to cause p.Ala277Val (11:2905355G>A; 1 in 107288 alleles) and p.Ala283Val (11:2905337G>A; rs776541692; 1 in 30726 alleles) changes. Of note, these codons are two of the lesser-conserved amino acids within this “hot-spot" region (Figure 2).

Expression of CDKN1C in human tissue
RNA-Seq analysis of CDKN1C in a panel of human tissues showed highest expression in the placenta (Figure 3; Human Protein Atlas data, https://www.proteinatlas.org/ENSG00000129757-CDKN1C/tissue), with strong expression also in adipose tissue, ovary, adrenal, endometri um and kidney. Immunohistochemistry in the Human Protein Atlas repository shows strong staining in the nuclei of both decidual and trophoblastic cells (https://www.proteinatlas.org/ENSG00000129757-CDKN1C/tissue/placenta).

Analysis of CDKN1C variants in growth restriction
Analysis of the PCNA-binding domain of CDKN1C by Sanger sequencing in a cohort of 58 children with isolated (non-familial) Silver-Russell syndrome did not reveal any pathogenic variants. Sequencing data for exon 2 is shown in Supplementary Data 1 (Achermann, 2018).

A next-generation sequencing approach of CDKN1C in children with IUGR/FGR (n=26), products of conception (n=21), and mothers with a history of recurrent miscarriage (n=71) also did not reveal pathogenic variants in this region (Table 1). Details of coverage for each sample/cohort and each nucleotide is shown in Supplementary Data 2–7 (Achermann, 2018).

Discussion
CDKN1C is now well-established as a key regulator of cell cycle and growth through G1 phase cell cycle arrest. Although loss-of-function of CDKN1C is known to cause macrosomia as part of Beckwith-Wiedemann Syndrome, it is only in the past six years that “gain-of-function" variants in CDKN1C have been shown to cause growth restriction and IMAGe syndrome. These findings demonstrate clearly how opposite effects in protein function can have opposite phenotypes (Arboleda et al., 2012; Eggermann et al., 2014). Sometimes these features affect not just growth but also endocrine systems (e.g. adrenal tumours (BWS)/adrenal hypoplasia (IMAGe); congenital hyperinsulinism (BWS)/diabetes mellitus (one family with growth restriction)).

Review of published literature confirms that CDKN1C SNVs associated with IMAGe syndrome or growth restriction are all located within a “hot-spot" of the PCNA-binding domain of the protein. The exact function of this region is not clear, and the crystal structure of this region of CDKN1C has not yet been solved. Studies to date suggest pathogenic variants may increase protein stability or reduce the rate of degradation, thereby enhancing the negative effects of CDKN1C on cell cycle...
progression (Borges et al., 2015; Hamajima et al., 2013). The very localised nature of these variants clearly demonstrates a key role for this region in CDKN1C function.

CDKN1C is strongly expressed in fetal adrenal development as shown by qPCR and immunohistochemistry (Arboleda et al., 2012), by microarray (Del Valle et al., 2017), and RNA-Seq (unpublished). Furthermore, analysis of RNA-Seq data from the Human Protein Atlas shows marked expression in the placenta (Figure 3). Immunohistochemistry shows strong nuclear staining in both decidual and trophoblastic cells. Therefore, gain-of-function of CDKN1C in the developing placenta could have a significant contribution to the growth restriction phenotype.

Further evidence for a potential role for CDKN1C in fetal growth restriction phenotypes has emerged with reports of CDKN1C variants in familial growth restriction and familial SRS (Brioude et al., 2013; Kerns et al., 2014). Several features of IMAGe syndrome and SRS overlap, such as bi-frontal bossing and relative micrognathia. To date these children have not shown evidence of adrenal insufficiency. Whilst a lack of adrenal features could be due to underlying mosaicism or a somatic “rescue” event, as recently reported for variants in SAMD9 in the related condition MIRAGE syndrome (Buonocore et al., 2017; Narumi et al., 2016), the fact that several individuals in a family with the same CDKN1C SNV were affected provides strong evidence that the primary genomic event is influencing the phenotype rather than a rescue mechanism. Of note, review of the two SNVs associated with FGR and normal adrenal function reveals that they affect amino acids at the C-terminal region of the hotspot. In one family a charged arginine at codon 279 is replaced by a non-polar leucine, whereas a proline at this position is found in classic IMAGe syndrome. In another family, the arginine at position 281 is replaced by a non-polar isoleucine. In some situations, variants in amino-acids flanking critical motifs can be associated with milder phenotypes (Kyriakakis et al., 2017).

Despite these findings, we did not identify any CDKN1C variants in children with isolated (non-familial) SRS or IUGR/ FGR. These results are similar to data from Brioude et al. who only reported a CDKN1C variant in familial SRS and did not find variants in 68 children with an isolated condition (Brioude et al., 2013). Whilst this does not exclude CDKN1C as a potential cause of isolated or sporadic SRS/FGR, it does suggest that it is not a common cause.

Identification of such strong expression of CDKN1C in the placenta lead us to consider whether gain-of-function/increased stability of CDKN1C could be associated with loss of pregnancy or recurrent miscarriage. This hypothesis was supported further by recent studies linking placental genes to pregnancy loss (Perez-Garcia et al., 2018); a potential role for placental CDKN1C expression in fetal growth and regulation by oestrogen (Chen et al., 2018; Gou et al., 2017; López-Abad et al., 2016; Unek et al., 2014); and the fact that paternal imprinting of the gene means that a pool of deleterious variants could be present in the population and cause pregnancy loss (together with live births) in women who carry this variant and inherit it from their father.

Analysis was therefore undertaken in a cohort of mothers who had recurrent miscarriages (often together with live birth(s)) and also from products of conception. However, no variants were found in the PCNA binding domain of CDKN1C in these cohorts.

This work has several limitations. Although 176 total individuals were studied, each sub group is still relatively small and rare CDKN1C variants might be discovered if the sample size is increased. The causes of recurrent miscarriage and fetal growth restriction are clearly diverse and can be influenced by many factors, and regulatory regions or enhancers (Perez-Garcia et al., 2018; Rai & Regan, 2006; Stalman et al., 2018; Wakeling et al., 2017). Nevertheless, this study does highlight the role of the key region of CDKN1C in human fetal growth restriction phenotypes, and starts to address the potential role of single gene growth restriction phenotypes in more common obstetric and fetal conditions.

Data availability
Data has been uploaded to OSF: http://doi.org/10.17605/OSF.IO/Y7KZV (Achermann, 2018). Representative sequencing data for chromatograms is shown in Supplementary Data 1. Data of coverage for next generation sequencing is shown in Supplementary Data 2–7.

Data are available under the terms of the Creative Commons Zero “No rights reserved” data waiver (CC0 1.0 Public domain dedication).

Access for samples from the Baby Bio Bank is available and can be requested from the steering committee by emailing the Baby Bio Bank Manager (nita.solanky@ucl.ac.uk). More information about data access can be found on the website under the ‘Protocol for the management of the Baby Bio Bank’, section 15: http://www.ucl.ac.uk/tapb/sample-and-data-collections-at-ucl/biobanks-ucl/baby-biobank/baby-biobank-documents/BBB_SOP_version7. An application form for the use of Baby Bio Bank resources must be completed prior to application (see appendix 2 in the protocol).

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