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RESEARCH ARTICLE

Analysis of *CDKN1C* in fetal growth restriction and pregnancy loss [version 1; peer review: 2 approved]

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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

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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 P57/kip2) (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/***I***ntrauterine growth restriction, ***M***etaphyseal dysplasia, ***A***drenal hypoplasia and ***Ge***nital anomalies (in males, usually relatively mild hypospadias 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 (also known as ICR1 or IC) 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.

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

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: CAGGAGCCTCTCGCTGAC; CDKN1CR2: GCTGGAGGGCACAACAAC). Polymerase chain reaction (PCR) was carried out with BIOTAQ 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 bcl 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

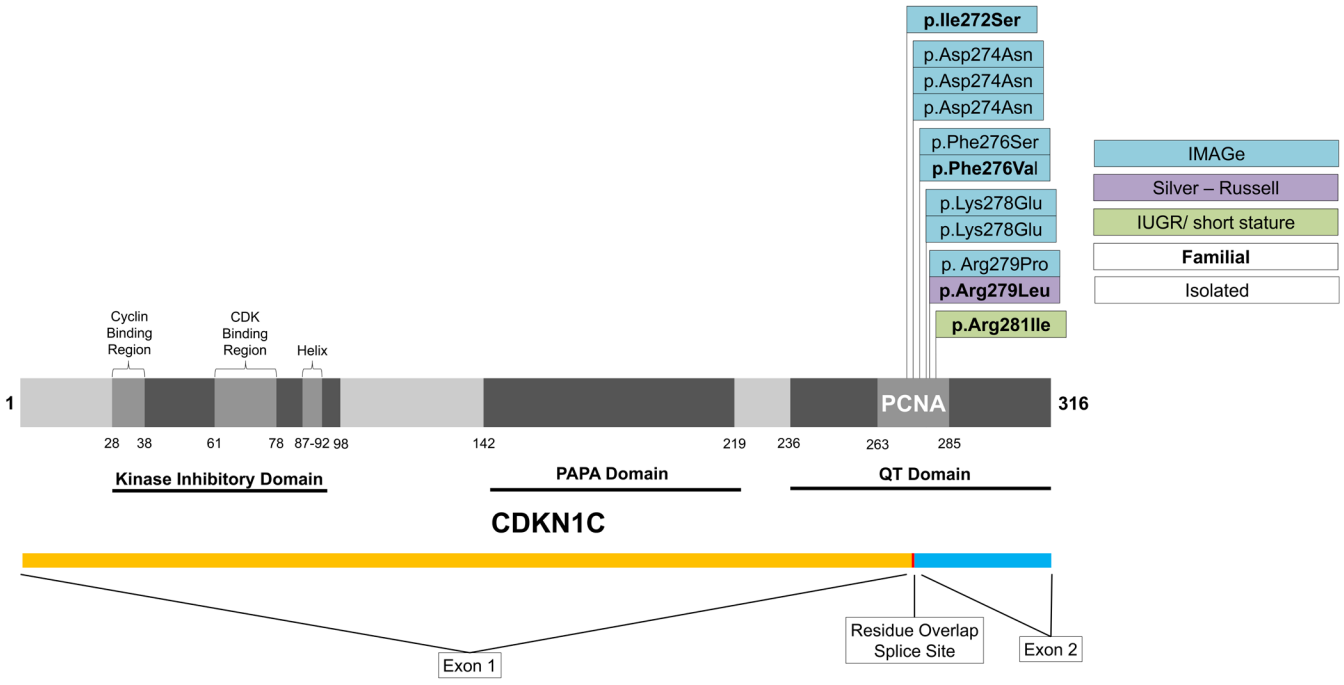

Figure 1. Schematic diagram showing the structure of CDKN1C and the clustering of pathogenic variants associated with IMAGe syndrome and/or growth restriction.

Table 2. Reported variants in CDKN1C and associated phenotypes.

Nucleotide variant	Protein change	Isolated/Familial	Phenotype	Reference
c.815T>G	p.Ile272Ser	Familial (3)	IMAGe	Hamajima *et al.*, 2013
c.820G>A	p.Asp274Asn	Isolated	IMAGe	Arboleda *et al.*, 2012
c.820G>A	p.Asp274Asn	Isolated	IMAGe	Kato *et al.*, 2014
c.820G>A	p.Asp274Asn	Isolated	IMAGe (Glucocorticoid)	Kato *et al.*, 2014
c.826T>G	p.Phe276Val	Familial (7)	IMAGe	Arboleda *et al.*, 2012
c.827T>C	p.Phe276Ser	Isolated	IMAGe	Arboleda *et al.*, 2012
c.832A>G	p.Lys278Glu	Isolated	IMAGe	Arboleda *et al.*, 2012
c.832A>G	p.Lys278Glu	Isolated	IMAGe (Mineralocorticoid)	Bodian *et al.*, 2014
c.836G>C	p.Arg279Pro	Isolated	IMAGe	Arboleda *et al.*, 2012
c.836G>T	p.Arg279Leu	Familial (9)	Silver-Russell	Brioude *et al.*, 2013
c.842G>T	p.Arg281Ile	Familial (15)	IUGR, short stature, IGT/DM	Kerns *et al.*, 2014

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

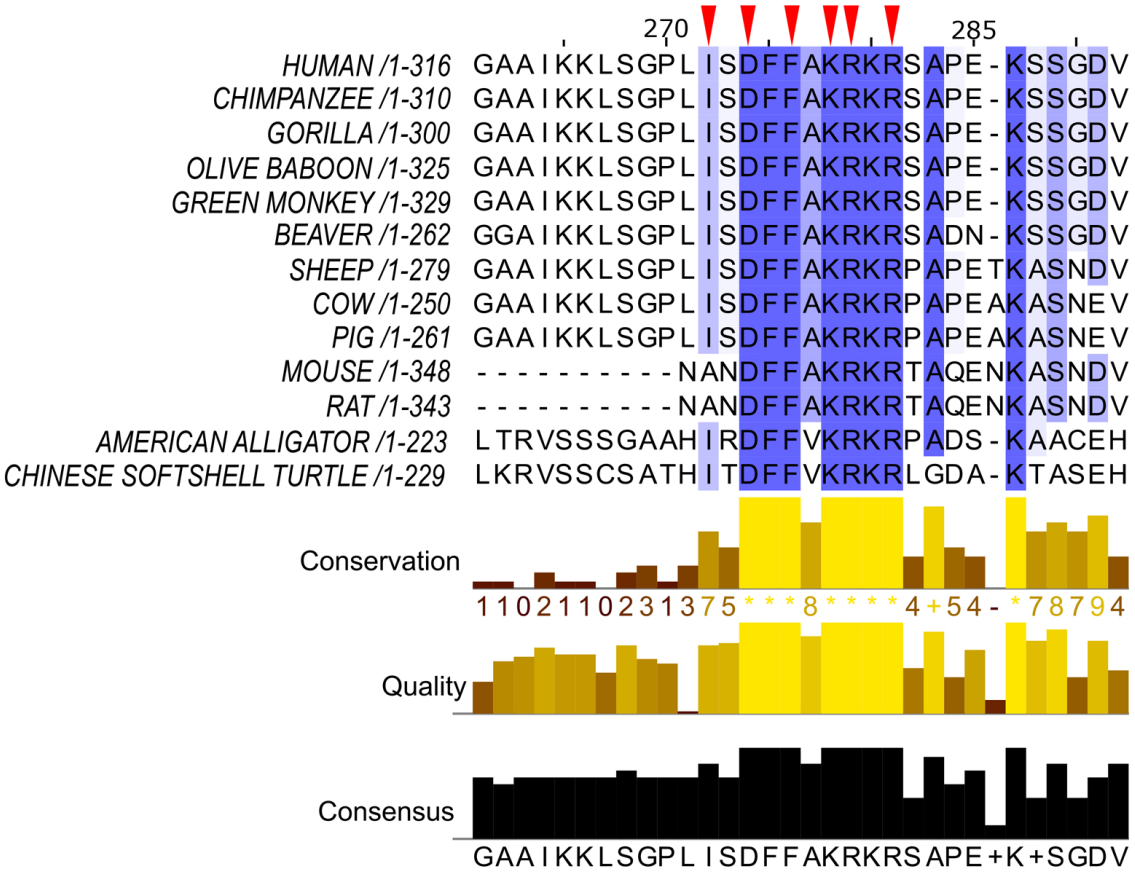

Figure 2. Amino-acid conservancy in the "hot-spot" region of CDKN1C. Red arrowheads represent codons that are mutated in IMAGe syndrome, FGR/IUGR or Silver-Russell syndrome. Yellow asterisks represent complete conservation amongst the species shown.

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, endometrium 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

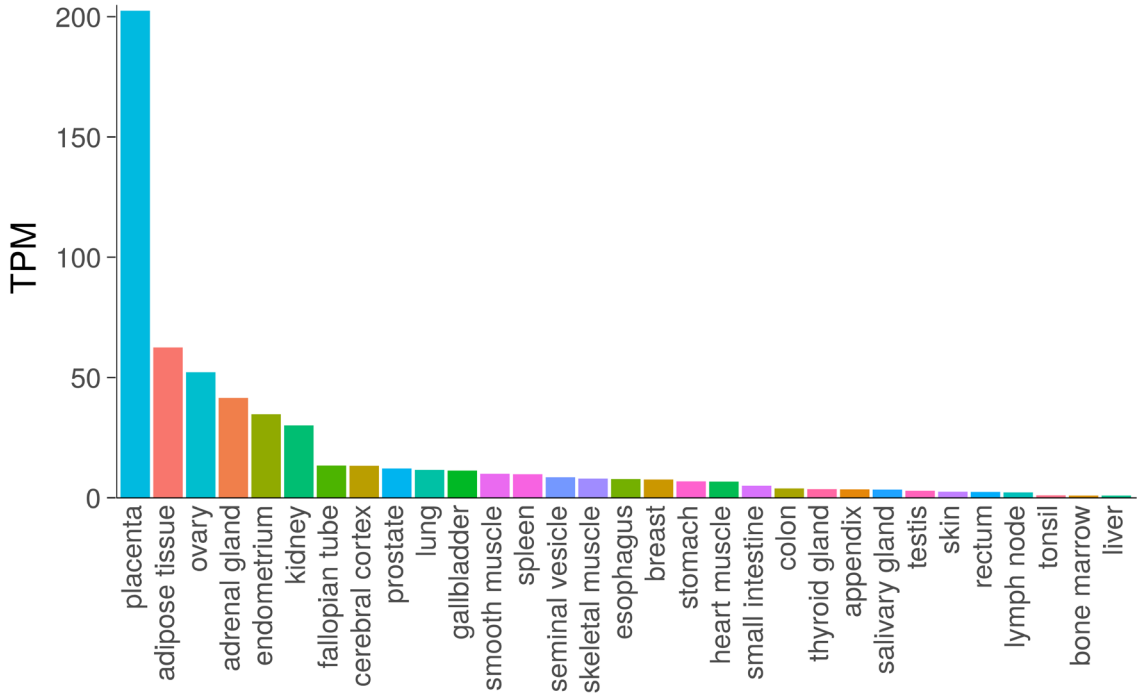

Figure 3. RNA expression of CDKN1C in placenta and different adult human tissues. Data reproduced and modified with permission from the Human Protein Atlas (www.proteinatlas.org) (Uhlen *et al.*, 2015). TPM = Transcripts Per Million.

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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In this report Suntharalingham and colleagues investigate the extent to which variations in a known negative regulator of cell growth (CDKN1C) may be involved in isolated growth restriction or recurrent pregnancy loss.

They approach this in several ways:

1. Through analysis of published literature

2. Through analysis of CDKN1C expression in multiple tissues using available RNA-seq data

3. Targeted sequencing in a number of patient populations.

As a result of the literature search, they conclude that reported variants are located in the PCNA-binding domain of CDKN1C and therefore focus on this for their sequencing analysis. The analysis of available RNA-seq data suggests that the gene is highly expressed in placenta, suggesting it may have a role in pregnancy maintenance. Sequencing analysis revealed no pathogenic variants in the targeted region of CDKN1C suggesting this is an uncommon cause of fetal growth restriction or recurrent pregnancy loss.

The paper is well written and easy to read, with appropriate citation of the literature.

Minor comments:

1. PubMed search terms appear quite limited. Are the authors certain they have not missed any papers by not including terms such as IUGR, FGR, SGA?

2. Do the pregnancies/children in the FGR/IUGR group have any other complications?

3. The term 'usually a history of live births' in table 2 is rather vague. Were any of these live births also affected by FGR/IUGR etc. or by pregnancy complications commonly associated with poor fetal growth e.g. pre-eclampsia.

4. In table 2 - do the numbers in brackets refer to the number of families with that variant or the number of affected individuals within a family?

Given the limited number of publications describing pathogenic mutations in the PCNA-binding domain of CDKN1C in IMAGe syndrome, familial SRS and in a single family with FGR and early adult onset diabetes, are the researchers planning to analyse other regions of the gene/its regulatory regions for other variations which may affect expression? Could they take this on to include analysis of placental gene expression/protein expression/function in assocision with analysis of sequence variation?

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Epigenetics, paediatrics, fetal growth, obstetrics

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 18 July 2019

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In this paper Suntharalingham and coworkers investigate the involvement of CDKN1C, a negative regulator of cell proliferation and growth, in fetal growth restriction (FGR)/intrauterine growth restriction (IUGR) human conditions, pregnancy loss or recurrent miscarriage. While many loss-of-function variants have been reported associated with the overgrowth condition Beckwith-Wiedemann syndrome and affecting all the domains of the protein, only few variants predicted to be “gain of function”, have been found associated with syndromic growth restriction conditions (IMAGe and SRS). The authors review the latter cases and find that all reported variants, associated with growth restriction, are localized in the same region inside the PCNA-binding domain, and affect evolutionary conserved amino acids.

By studying RNA-seq data available on the Human Protein Atlas website, the authors consider the high expression level of *CDKN1C* in placenta to hypothesize its involvement in pregnancy loss and miscarriage as a consequence of placenta restriction. Following a DNA targeted sequencing analysis restricted to the “hot spot” PCNA-binding domain in isolated cases of SRS (n=58), IUGR/FGR (n=26), loss of pregnancy (n=21) and women with recurrent miscarriage (n=71), they do not find any pathogenic variant, concluding that pathogenic variants of this analysed region are not a common cause of these pathological conditions.

The paper is interesting and easy to read. The potential role of CDKN1C in pregnancy maintenance and its putative involvement in pregnancy loss is interesting, and an aspect that in future could be better investigated by extending the number of the cases to analyze.

It would be interesting if the authors could report the analysis of the variants extended to the CDKN1C domains other than the region corresponding to the “hot spot” PCNA-binding domain (at least for cases analysed by NGS).

Minor comments:

- The authors should clarify why they have analyzed *CDKN1C* variants only in the isolated SRS cases, excluding the familial cases.

- Most of information reported in Table 2 are already present in Figure 1, therefore Table 2 could be removed or reported as Supplementary Table.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Genomic imprinting, Imprinting disorders

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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