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HAL Id: hal-01737991

https://hal.sorbonne-universite.fr/hal-01737991

Submitted on 20 Mar 2018

Genetic disruption of the oncogenic *HMGA2–PLAG1–IGF2* pathway causes fetal growth restriction

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Purpose: Fetal growth is a complex process involving maternal, placental and fetal factors. The etiology of fetal growth retardation remains unknown in many cases. The aim of this study is to identify novel human mutations and genes related to Silver–Russell syndrome (SRS), a syndromic form of fetal growth retardation, usually caused by epigenetic downregulation of the potent fetal growth factor IGF2.

Methods: Whole-exome sequencing was carried out on members of an SRS familial case. The candidate gene from the familial case and two other genes were screened by targeted high-throughput sequencing in a large cohort of suspected SRS patients. Functional experiments were then used to link these genes into a regulatory pathway.

Results: We report the first mutations of the *PLAG1* gene in humans, as well as new mutations in *HMGA2* and *IGF2* in six

sporadic and/or familial cases of SRS. We demonstrate that HMGA2 regulates *IGF2* expression through PLAG1 and in a PLAG1-independent manner.

Conclusion: Genetic defects of the *HMGA2–PLAG1–IGF2* pathway can lead to fetal and postnatal growth restriction, highlighting the role of this oncogenic pathway in the fine regulation of physiological fetal/postnatal growth. This work defines new genetic causes of SRS, important for genetic counseling.

Genet Med advance online publication 10 August 2017

Key Words: fetal growth restriction; HMGA2; IGF2; PLAG1; Silver–Russell syndrome

INTRODUCTION

Intrauterine growth retardation (IUGR) is a common condition arising from multiple origins (environmental, (epi)genetic, vascular, etc.).[1] A well-characterized syndromic form of IUGR is represented by Silver–Russell syndrome (SRS, OMIM 180860). SRS is a clinically and genetically heterogeneous imprinting disorder characterized by fetal and postnatal growth retardation, relative macrocephaly at birth, a prominent forehead, and additional features.[2] The syndrome remains a clinical diagnosis, defined by clinical scoring systems.[2–6] *Insulin-like growth factor 2* (*IGF2*) is a growth factor strongly implicated in fetal growth and in the pathophysiology of SRS, as up to 50–60% of patients display hypomethylation of the imprinted 11p15.5 *IGF2/H19* domain, which leads to the downregulation of *IGF2* expression.[2,7,8] The second most common cause of SRS is maternal uniparental disomy of chromosome 7, accounting for about 10% of cases.[2] Other rare 11p15.5-related genetic defects, such as maternal duplications, maternal *CDKN1C* mutation,[9] and

paternal *IGF2* point mutation[10] have also been implicated in SRS. In about 30–40% of patients with a clinical diagnosis of SRS, the molecular etiology of this syndrome remains unknown, involving molecular mechanisms and genes other than those cited above.[6]

Pleomorphic adenoma gene 1 (*PLAG1*), on human chromosome 8q12, was initially identified as an oncogene associated with certain types of cancer (e.g., pleomorphic adenomas, lipoblastoma, hepatoblastoma).[11–13] The tumorigenic capacity of *PLAG1* results from its ability to bind the P3 promoter of *IGF2,* thereby increasing *IGF2* expression in tumor cells.[13–16] In addition to functioning as an oncogene, *PLAG1* has been implicated in growth, as *Plag1* knockout mice and paternal *Igf2*-deficient mice have remarkably similar phenotypes, characterized by intrauterine and postnatal growth retardation.[17]

The DNA-binding protein *high-mobility group AT-hook 2* (*HMGA2*), located on human chromosome 12q14, is a transcription factor that has been identified as an oncogene

and shown to be an upstream regulator of *PLAG1* expression in several tumor cells and experimental models.[18] *Hmga2*-deficient mice have a pygmy phenotype and fetal growth retardation.[19] A splicing mutation of *HMGA2* was recently associated with SRS in a family but the contribution of this mutation to the phenotype was not clearly assessed.[20]

Rearrangements of 8q12 and 12q14 chromosomal regions corresponding to the locations of *PLAG1* and *HMGA2*, respectively, have been reported in patients with SRS and SRS-like conditions.[21] Finally, GWAS meta-analysis studies on humans have shown *HMGA2* and *PLAG1* variants to be strongly correlated with childhood/adult height, highlighting the role of these genes in the control of human growth.[22–25]

Together, these observations strongly suggest that *HMGA2* and *PLAG1* play a role in growth physiology mediated by IGF2, but direct evidence has been lacking.

Here we report new mutations in the *HMGA2*–*PLAG1*–*IGF2* pathway resulting in lower levels of *IGF2* expression in SRS patients. These findings highlight the role of HMGA2 and PLAG1 as upstream regulators of *IGF2*.

MATERIALS AND METHODS
Population studied
The familial case and the 192 patients included in this study were referred for IUGR and suspected Silver–Russell syndrome. Blood samples were collected during clinical visits.

Written informed consent for participation was received from all patients or parents, in accordance with national ethics rules (Assistance Publique–Hôpitaux de Paris authorization no. 681 for French patients and IRB I00000204 at the Mount Sinai School of Medicine, New York, for patients recruited in the United States). Written consent was also obtained to publish patient photos. Patients were either followed at Armand Trousseau Children's Hospital or referred by other clinical centers for molecular analysis of suspected SRS. A geneticist and/or a pediatric endocrinologist examined each patient and a detailed clinical form was completed. Patients who were negative for methylation defects within the imprinted domains controlling *IGF2/H19*, *DLK1/GTL2*, and *MEST/GRB10* assessed by allele-specific methylated multiplex real-time quantitative polymerase chain reaction, as previously described,[2] were retained for further molecular analysis.

Whole-exome sequencing and targeted *HMGA2*–*PLAG1*–*IGF2* sequencing
Library preparation, exome capture/gene enrichment, sequencing, and data analysis were performed by IntegraGen SA (Evry, France). The sequencing methods and bioinformatics analysis are described in detail in the **Supplementary Methods** online.

Sanger sequencing and short tandem repeat typing
HMGA2, *PLAG1,* and *IGF2* mutations identified with whole-exome sequencing and targeted sequencing were verified by standard methods of Sanger sequencing, using the ABI PRISM Big Dye Terminator v3.0 Cycle Sequencing Kit and

an ABI 3100 Genetic Analyzer (Life Technologies, Courtaboeuf, France). The sequencing products were then analyzed with SeqScape v2.6 (Life Technologies). For de novo mutations, parental inheritance was verified using short tandem repeat typing on chromosome 14 (D14S65 and D14S292).

Cell cultures and transfections
Fibroblasts, Hep3B, and HEK293 cells were cultured under standard conditions in supplemented RPMI 1640 and MEM, respectively (Gibco, Cergy Pontoise, France), at 37 °C. Gene silencing and overexpression assays were performed according to the manufacturer's protocols (Thermo Fisher, France). Details of the culture conditions, gene silencing, and overexpression assays as well as RNA extraction are provided in the **Supplementary Methods**.

Reverse transcription and real-time messenger RNA quantification
We generated complementary DNA from the messenger RNA of fibroblasts, Hep3B, and HEK293 cells, with the SuperScript II reverse transcription system (#18064-014 Invitrogen Thermo Fisher, France). Real-time PCR was performed on the complementary DNAs obtained, in an ABI-7900HT machine, and gene expression was quantified with the Power SYBR Green PCR Master Mix (Life Technologies). Details from the reverse transcription and real-time quantification analysis are provided in the **Supplementary Methods**.

Statistical analysis
We compared data for pairs of groups in Mann–Whitney (expression in fibroblasts) and unpaired *t* (silencing and overexpressing assays) tests. We considered *P* values of less than 0.05 to indicate statistical significance. The analyses were performed with GraphPad Prism version 6.00 (GraphPad Software, La Jolla, CA).

RESULTS
Heterozygous *PLAG1* frameshift mutation
SRS was diagnosed clinically using the Netchine–Harbison clinical scoring system (NH–CSS)[2,6] in patient II-2 from the affected family (**Figure 1**). Molecular analysis revealed a normal methylation on chromosomes 11, 7, and 14. Single-nucleotide polymorphism array excluded maternal uniparental disomies and chromosomal rearrangements. The proband's sister and mother had similar phenotypes consistent with dominant transmission of the disease. Whole-exome sequencing revealed a heterozygous deletion of a single nucleotide within exon 5 of the *PLAG1* gene (NM_002655.2: c.439del) in the proband, her mother, and her sister, but not in the father. This deletion leads to a frameshift and production of a truncated, 227–amino acid peptide (NP_002646.2:p.Ser147Valfs*82). This variant was not reported in any polymorphism or the ExAC database. The 18 other variants listed in **Supplementary Table 1**, which were found in the three affected family members, have no known effect on growth.
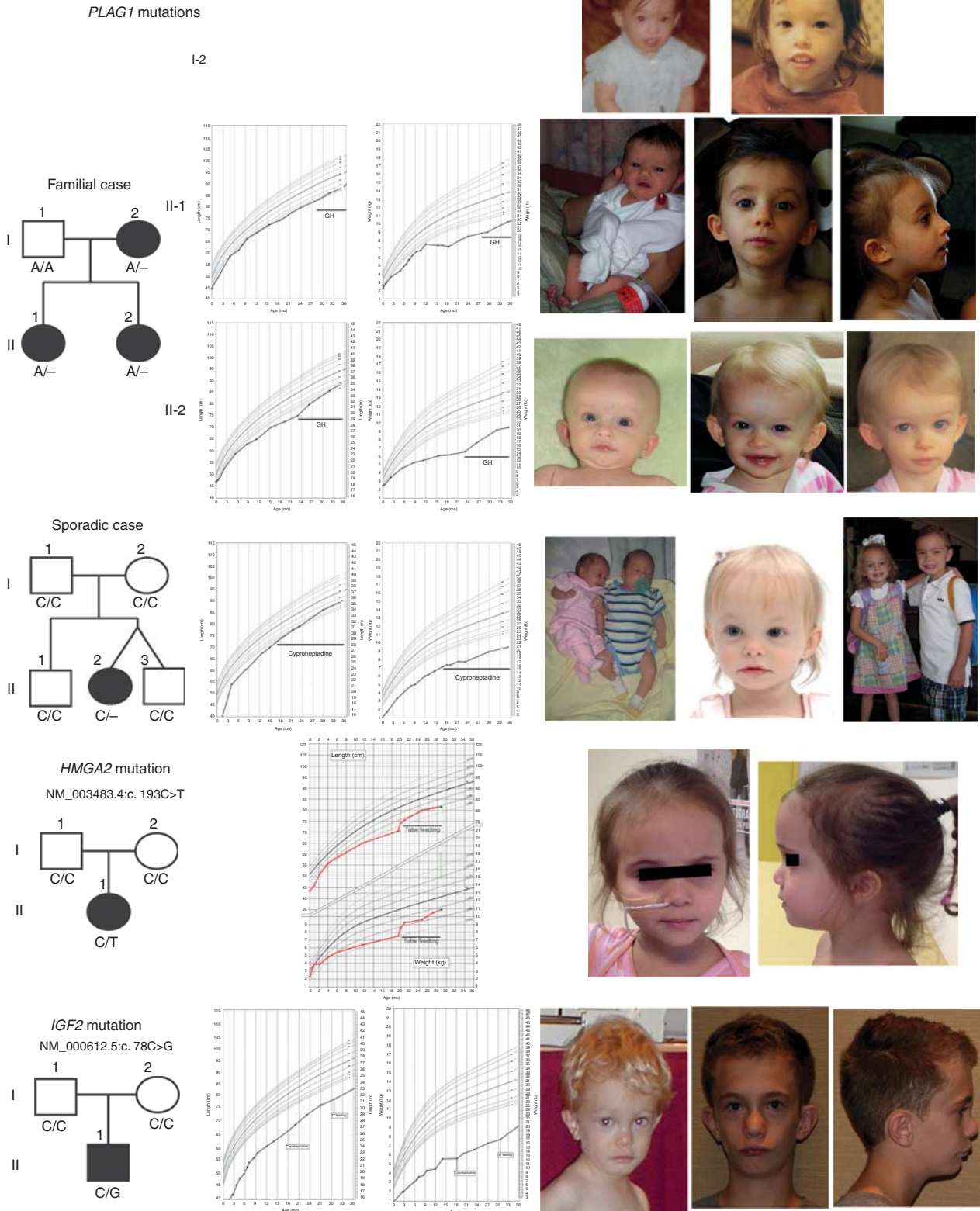
Figure 1 Pedigrees, growth charts, and photographs of patients carrying mutations affecting the *HMGA2–PLAG1–IGF2* pathway. The patient with sporadic *PLAG1* anomaly is next to her healthy twin brother. The patient with *IGF2* anomaly shows that a prominent forehead evident in early childhood may become less obvious in later life. The patients' growth charts between the ages of 2 and 20 years are included in the **Supplementary Figure**.

Description of the screened population

We have selected 192 patients for whom a diagnosis of SRS was suspected and for whom we could not identify any known epigenetic or genetic cause of SRS. All these patients were born small for gestational age (birth weight and/or birth length < −2 standard deviation score (SDS)) and without body asymmetry, which is in favor of a genetic rather than an epigenetic defect. Of these patients, 76 displayed head circumference sparing with relative macrocephaly at birth.

Identification of new genetic defects affecting the HMGA2–PLAG1–IGF2 pathway

As *PLAG1* plays a key role in the *HMGA2–PLAG1–IGF2* pathway, we screened for mutations using high-throughput targeted sequencing for these three genes in the cohort of patients described above. We identified another heterozygous, de novo deletion of a single nucleotide in *PLAG1* (NM_002655.2:c.1363del), leading to a frameshift and the production of a truncated, 469–amino acid protein (NP_002646.2:pGln455Serfs*16). This deletion was not found in the parents, the twin brother, or the older brother of the patient (**Figure 1**). Two new heterozygous variants were identified in the *HMGA2* gene: a de novo nonsense mutation (NM_003483.4:c.193C > T) leading to premature termination (NP_003474.1:p.Gln65*), and a deletion of a single nucleotide (NM_003483.4:c.189del) leading to an elongated protein (NP_003474.1:p.Ala64Leufs*102) (**Table 1**). Due to lack of parental DNA, the origin of this deletion could not be investigated, but the father's adult height of 155 cm strongly suggests that he passed this deletion to his son. Finally, we found two heterozygous variations within the *IGF2* gene: a de novo nonsense mutation (NM_000612.5:c.78C > G) leading to the production of a truncated peptide (NP_000603.1:p.Tyr26*), and a de novo two-nucleotide duplication (NM_000612.5:c.158_159dup) leading to a frameshift and the production of a truncated 59–amino acid protein (NP_000603.1:p.Arg54Alafs*7).

All five mutations were identified in the group of 76 patients with relative macrocephaly at birth.

Clinical data

The clinical presentation of the patients harboring the mutations is summarized in **Table 1**. We were unable to categorize patient I-2 due to a lack of information about some of her clinical signs. For the remaining patients, five out of seven fulfilled the SRS clinical diagnostic criteria of the NH–CSS (at least four of six criteria, including relative macrocephaly). Patient II-1 fulfilled only three of the six criteria from the NH-CSS and patient II-2 fulfilled four of six criteria without relative macrocephaly at birth. Birth weights and lengths were most strongly affected for patients with *IGF2* and *HMGA2* mutations (respectively), with a slightly smaller effect observed in patients with *PLAG1* defects. Similarly, relative macrocephaly, defined as a difference in SDS of at least 1.5 between head circumference and length or weight at birth, was more pronounced in patients with the *IGF2*

mutations. As is characteristic of SRS, all patients presented with significant feeding difficulties and need for nutritional support with nonvolitional feeding and/or appetite stimulation. Serum IGF2 levels and other related markers from the patients with *PLAG1, HMGA2,* and *IGF2* mutations are listed in **Table 1**.

Regulation of IGF2 levels by HMGA2 and *HMAG2–PLAG1* pathway

The small interfering RNA (siRNA)-mediated silencing of *HMGA2* in Hep3b cells decreased *PLAG1* and total *IGF2* expression (**Figure 2a**). Silencing of *PLAG1* resulted in lower levels of total *IGF2,* but did not change the levels of *HMGA2* expression (**Figure 2a**). Since PLAG1 binds specifically to the *IGF2* promoter P3, we assessed the levels of *IGF2* generated by transcription from the P1 and P3 promoters (*IGF2*-P1 and *IGF2*-P3, respectively). The silencing of both *HMGA2* and *PLAG1* led to a downregulation of *IGF2*-P3, whereas *IGF2*-P1 expression remained normal (**Figure 2b**). Finally, *H19* expression was not affected by the silencing of either *HMGA2* or *PLAG1*. PLAG1 therefore acts as a *trans*–acting factor at the *IGF2*-P3 promoter, but does not affect the whole *IGF2/H19* domain (**Figure 2a**). *IGF2*-P3 levels were significantly lower in si*HMGA2*-treated than in si*PLAG1*-treated cells despite the production of significantly lower levels of PLAG1 in si*PLAG1*-treated cells (**Figure 2a,b**), suggesting a PLAG1-independent HMGA2 regulation of *IGF2*. In order to verify this, we performed an overexpressing assay of *HMGA2* and *PLAG1* in HEK293 cells. Overexpression of *HMGA2* in these cells resulted in an increased expression of *IGF2*-P3, without affecting the messenger RNA levels of *PLAG1* (**Figure 2c**). However, PLAG1 maintained its capacity to upregulate *IGF2*-P3 upon *PLAG1* overexpression (**Figure 2c**). These results show that HMGA2 and PLAG1 both positively regulate *IGF2*-P3 expression, independently or via a *HMGA2–PLAG1–IGF2* pathway.

We further investigated gene expression in fibroblasts from patient I-2 from the family (**Figure 1**) carrying the *PLAG1* deletion. The levels of *PLAG1* expression were similar to those in controls, but *IGF2* expression was half that of the controls (**Figure 2d**).

DISCUSSION

IGF2 has been identified as a major factor in the control of fetal growth in many species, including humans. *IGF2* is a maternally imprinted gene, and its expression is regulated mostly by epigenetic marks.[27] Epigenetic and genetic defects within 11p15.5 have been implicated in syndromes involving growth retardation (SRS, with *IGF2* downregulation)[28] or overgrowth (Beckwith–Wiedemann syndrome, with *IGF2* overexpression).[29] Furthermore, the overexpression of *IGF2* is frequently observed in several types of tumors, including embryonal tumors in particular.[30] The epigenetic regulation of *IGF2* expression is well described, but the upstream genetic mechanism by which *IGF2* expression is regulated remains unknown.

Table 1 Clinical features of patients with Silver–Russell syndrome and mutations of the *PLAG1*, *HMGA2*, or *IGF2* genes

GENE	PLAG1				HMGA2		IGF2	
Case	Familial case patient I-2	Familial case patient II-1	Familial case patient II-2	Sporadic case	Sporadic case	Sporadic case	Sporadic case	Sporadic case
Defect	NM_002655.2:c. 439del	NM_002655.2:c. 439del	NM_002655.2:c. 439del	NM_002655.2:c. 1363del	NM_003483.4: c.193C > T	NM_003483.4: c.189del	NM_000612.5:c. 78C > G	NM_000612.5:c. 158_159dup
Effect and prediction	Frameshift- premature stop Deleterious	Frameshift-premature stop Deleterious	Frameshift-premature stop Deleterious	Frameshift-premature stop Deleterious	Premature stop Deleterious	Frameshift-elongated protein Deleterious	Premature stop Deleterious	Frameshift-premature stop Deleterious
ClinVar accessions	SCV000297812	SCV000297812	SCV000297812	SCV000297813	SCV000297814	SCV000297815	SCV000297816	SCV000297817
Gender	Female	Female	Female	Female	Female	Male	Male	Female
Phenotype	SRS adult	SRS	SRS	SRS	SRS	SRS	SRS	SRS
Gestational age (weeks of amenorrhea)	Term	39 w 6 d	39 w	31 w 6 d	39	37	32 w 2 d	29
Birth length cm/SDS	**Very small**	**44.5/–2.3**	**47/–2**	**37.5/–2.78**	**43.5/–3.9**	**40/–4.8**	**34.5/–4.97**	**30/–5.5**
Birth weight g/SDS	**Very light**	**2,340/–2.1**	2,557/–1.9	**1,050/–3**	**2,300/–2.5**	**1,720/–3.1**	**960/–3.57**	**570/–4**
Birth head circumference cm/SDS[a]	?	31/–3.84	31/–3.45	27.4/–1.5	32.5/–2	31/–2.3	27/–2.13	23.5/–2.3
Relative macrocephaly at birth	?	No	No	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
Length cm at 2 years cm/SDS	**Very small**	**79/–2.0**	**75/–2.8**	**79/–2.0**	**66/–3.4**	**91.5/–3 (4 yr)**	**73.4/–3.5**	**65/–5.1**
Weight g at 2 years/SDS	Very light	8,520/–3.7	6,600/–6.7	7,800/–4.4	6,850/–3.6	11,800/–4 (4 yr)	6,590/–6.1	5,370/–6.2
BMI at 2 years/SDS	?	**13.5/–2.3**	**11.7/–3.8**	**12.5/–3.2**	15.6/–0.6	14.1/–1.1	**12.2/–3.5**	**12.5/–3.2**
Feeding difficulties during infancy	?	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
Prominent forehead during infancy	**Yes**	No	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
Body asymmetry	No	No	No	No	No	No	No	No
N–H score	3/4[b]	3/6	4/6	4/6	5/6	5/6	5/6	5/6
Head circumference cm/SDS 2 yr[a]	51.5/SDS –2.8 (adult)	43.9/–2.6	43.3/–2.5	44.7/–2.0	44.5/–1.2	NA	46.4/–1.7	43.2/–3.1
Triangular facies	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Final height cm/SDS	148.3/–2.31	NA	NA	NA	NA	NA	NA	NA
GH therapy and age at start	No	Yes: 2.5 yr	Yes: 2 yr	Yes: 6 yr	No	Yes: 4.2 yr	Yes: 3.3 yr	Yes: 4.4 yr
GI/feeding therapies	No	Cyproheptadine[c]	Cyproheptadine[c]	Cyproheptadine[c]	NGT 1.6 yr	GT	Cyproheptadine[c] GT 2.6 yr	NGT 2 months
Bsl IGF1 (ng/ml)/SDS	ND	162/+2.8	67/–1.5	119/-1.2	ND	142/+1.3	152/+3.30	285/+5.4
Bsl IGFBP3 (mg/L) [R 0.8–3.0 M 2.1]/ALS/SDS	ND	2.6/20/+5.8	2.5/10/+1.8	2.1/8.3/+0.4	ND	ND	2.0/ND/ND	ND
GH dose (mg/kg/wk)/ IGF1/SDS	ND	0.26/368/+7.2	0.27/195/+3.1	0.24/255/+1.7	NA	0.31/402/+5	0.34/525/+9.0	0.36/348/+4.5
GH-treated IGFBP3 [R 1.5–3.4 M 2.4]/ALS/SDS	ND	3.5/19/+4.5	3.0/16./+3.4	3.4/14/+2.8	NA	3.2/ND/NA	5.3/15/+2.4	ND
IGF2 (ng/ml)	ND	498[d]	393[d]	299[d]	ND	920[d]	486[e] and 382[e]	ND

ALS, acid labil subunit; BMI, body mass index; Bsl, basal; GH, growth hormone, GI, gastrointestinal; IGF, insulin-like growth factor; NA, not applicable; ND, not determined; (N)GT, (naso)gastric tube; N–H, Netchine–Harbison; SDS, standard deviation score; SRS, Silver–Russell syndrome.
The criteria of the Netchine–Harbinson scoring system are marked in bold characters.
[a]Head circumference at birth ≥ 1.5 SDS above birth weight and/or length SDS. [b]For this adult patient, the Netchine–Harbison clinical scoring system was determined from four criteria only, as the two additional items (birth head circumference and BMI at 2 years) were not available. [c]Cyproheptadine, a first-generation antihistamine, has also been used as an orexigenic drug in some pediatric disorders with malnutrition.[26] [d]IGF2 Mediagnost RIA assay, 3–7 years normal range 397–973 ng/ml. [e]IGF2 Esoterix assay, prepubertal normal range 334–642 ng/ml.
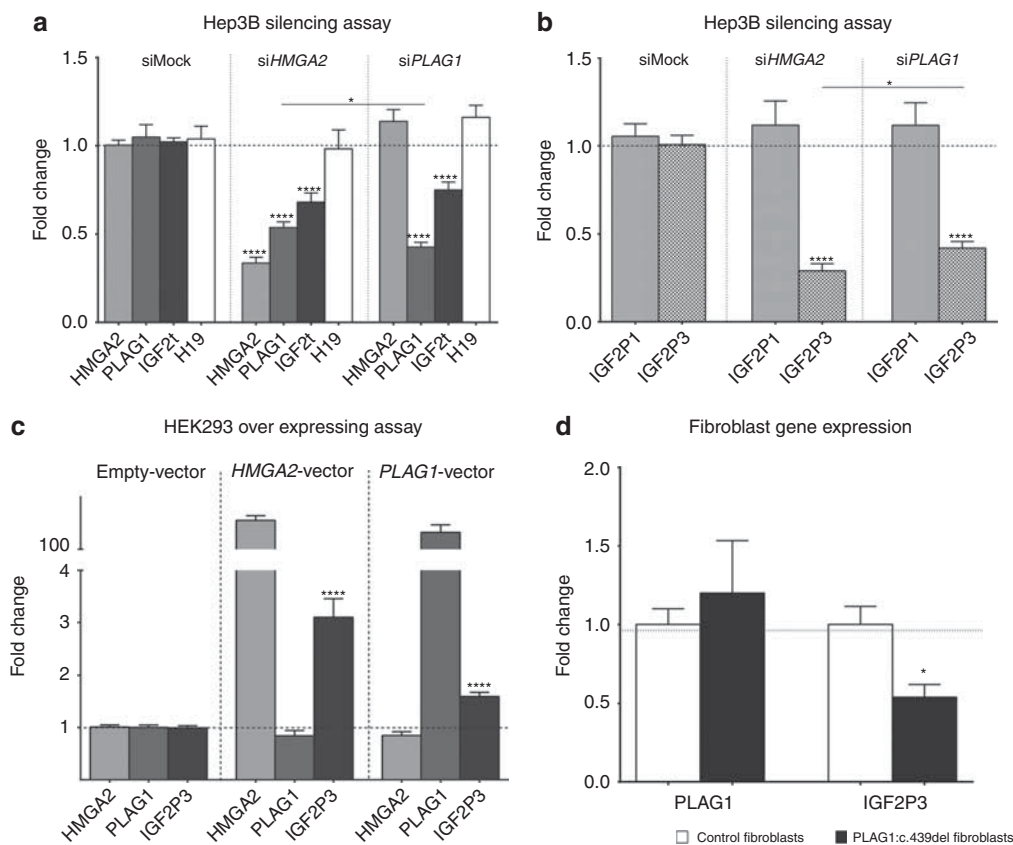
Figure 2 Functional characterization of the effects of *HMGA2* and *PLAG1* silencing/mutation on *IGF2* expression. (**a**) HMGA2 is an upstream regulator of *PLAG1*, which in turn is an upstream regulator of *IGF2*. (**b**) The HMGA2–PLAG1 pathway regulates *IGF2*-P3 expression. (**c**) The *IGF2* regulation by HMGA2 in a PLAG1-independent manner. (**d**) The effect of the PLAG1:c.439del on IGF2 expression in cultures of patient fibroblasts. A single asterisk indicates a $P < 0.05$, and four asterisks indicate a $P < 0.0001$. The histograms represent the fold change means of biological replicates. Three separate small interfering RNA (siRNA) and vector transfection assays were performed. Each transfection assay contained at least four transfected wells of control and siRNA or vector transfection ($N \geq 12$ biological sample per group per assay). For fibroblast expression, five cultures of control fibroblasts derived from different donors were compared to five different passages of cultured fibroblasts from the patient carrying the PLAG1 single-nucleotide deletion. The error bars indicate the standard error of the mean.

We report the first mutations of *PLAG1* in a familial case with dominant transmission of syndromic IUGR associated with SRS and in another sporadic case of SRS with a de novo mutation. Since PLAG1 is a key factor in the *HMGA2–PLAG1–IGF2* pathway, we screened for and identified mutations of the *HMGA2* and *IGF2* genes. These findings highlight the crucial role of this pathway in controlling fetal IGF2 levels and its involvement in the pathophysiology of IUGR and SRS (**Figure 3**).

These observations also demonstrate that, in addition to epimutation at the 11p15 locus and mutation in the imprinted *IGF2* gene, SRS may be caused by mutations of genes controlling *IGF2* expression, such as *PLAG1* and *HMGA2*. Though previously shown in mice,[31] this is one of the first examples in humans, where nonimprinted genes deregulate the expression of distant, normally imprinted genes.

The mutations we identified in *PLAG1* and *HMGA2* were heterozygous leading to dominant transmission of SRS. The

mutations we found in *IGF2* were also heterozygous, but because *IGF2* is paternally expressed in most tissues during fetal and postnatal development, to induce the SRS phenotype, an *IGF2* mutation must occur on the paternal allele. We could not find any other inherited polymorphism near the de novo mutations in the *IGF2* gene to specifically determine the parental origins of these mutations. However, given the typical SRS phenotype of these reported patients who carry the de novo *IGF2* mutations, it is very likely that the mutations affected their paternal alleles, consistent with the imprinted status of this gene.

Very few mechanistic data on the *HMGA2–PLAG1–IGF2* pathway or its role in pathological processes have been reported to date. All previous studies have shown *HMGA2–PLAG1* and *PLAG1–IGF2* expression levels to be correlated, in overexpression models or in tumors.[18,32] Murine models with inactivation of *Hmga2*,[19] *Plag1*,[17] or *Igf2*[33] display growth retardation, but the processes underlying the pathological phenotype have yet to be determined.
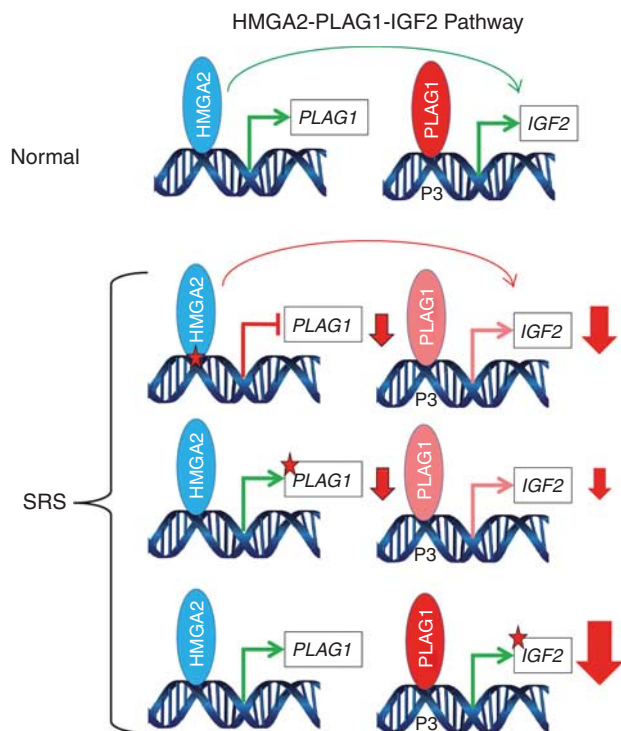
Figure 3 Schematic representation of the *HMGA2–PLAG1–IGF2* pathway in a normal individual and in Silver–Russell cases. Green arrows indicate normal expression, the sizes of red arrows are representative of *IGF2* downregulation and red stars indicate functional impairment for *HMGA2*, *PLAG1*, or *IGF2* expression. SRS, Silver–Russell syndrome.

We show that the disruption of any genes in this pathway leads to a decrease in *IGF2* expression and produces a SRS phenotype, similar to patients carrying 11p15.5 epigenetic defects. We also show that this downregulation of *IGF2* expression is caused by a lack of activation of the *IGF2* P3 promoter, consistent with the results of previous studies showing a direct interaction between PLAG1 and this promoter.[34] *IGF2*-P3 is the main transcript and is highly active in fetal tissues, whereas *IGF2*-P1 is active only in the liver after birth.[35] This specific action at *IGF2*-P3 may account for the nondecreased levels of IGF2 in patients with mutations of the *PLAG1* and *HMGA2* genes. Indeed, circulating IGF2 is synthesized from the P1 promoter in a biallelic fashion by the adult liver in humans with no imprinting in this organ.[36] The silencing of *HMGA2* or *PLAG1* had no effect on *IGF2* transcription from the P1 promoter. Therefore, patients with an *HMGA2–PLAG1* defect display conserved P1 transcription activity in the liver, resulting in normal postnatal IGF2 serum levels. The patients with the *IGF2* mutations had low normal or modestly decreased levels of IGF2 in the serum (**Table 1**). In this case, the circulating IGF2 was probably produced by transcription from the unaffected allele alone. This is in accordance with the modestly decreased circulating IGF2 levels observed in the previously described patients carrying paternal *IGF2*[10] mutations (**Table 1**).

As expected with a genetic defect, none of the cases have body asymmetry. In a family carrying this kind of mutation, body asymmetry is not expected to occur because the molecular defect is present in all the cells of the body (unlike the mosaic epigenetic change at the 11p15.5 locus). Thus, the absence of body asymmetry does not exclude the diagnosis of SRS in this particular case. However, despite the fact that patients harboring a genetic defect of the *HMGA2–PLAG1–IGF2* pathway presented IUGR and an SRS clinical diagnosis, some differences were observed between the phenotypes. Indeed, patients with the *IGF2* and *HMGA2* defects were smaller at birth and their head circumferences were larger than those patients with *PLAG1* defects. These differences between the phenotypes could simply reflect individual human variations, and more cases are needed to confirm these observations. Such variability is also observed within the group of SRS patients with *IGF2/H19* hypomethylation. However, using siRNA-based silencing technique in cell lines, we showed that *HMGA2* silencing led to significantly lower levels of *IGF2* expression than *PLAG1* silencing, despite a more effective *PLAG1* downregulation in the latter silencing assay. Furthermore, using vector-based overexpressing technique in another cell line, we showed that HMGA2 is capable of regulating *IGF2* expression in a PLAG1-independent manner. Thus, *IGF2* expression is more strongly affected by *IGF2* and *HMGA2* mutations, respectively, than by *PLAG1* mutations.

The elucidation of these new genetic mechanisms has major implications for genetic counseling. In keeping with the imprinted, paternally expressed nature of the gene, males carrying *IGF2* mutations have a 50% risk of transmitting the disorder. As *HMGA2* and *PLAG1* are not imprinted, males and females with mutations of these genes both have a 50% risk of transmitting SRS. Given this high risk of SRS transmission, screening for mutations of these genes should be considered in cases of SRS presenting with no epigenetic defect of 11p15.5 or maternal disomy of chromosome 7. Furthermore, in the context of next-generation sequencing, these genes could be included in panels for the screening of growth retardation disorders for patients with no 11p15 epimutations or maternal disomy of chromosome 7 or 14.

In conclusion, we show for the first time that defects of the *HMGA2–PLAG1–IGF2* pathway can lead to SRS, highlighting the role of this oncogenic pathway in the fine regulation of physiological fetal/postnatal growth and food intake. We also show that nonimprinted genes can deregulate the expression of distant imprinted genes in humans. Given the crucial role played by the low level of *IGF2* expression in the outcome of IUGR and the SRS phenotype, identification of new targets of *IGF2* will be the next step in the development of new treatment options for this group of patients.

Accession numbers
The ClinVar accession numbers for the *PLAG1*, *HMGA2*, and *IGF2* variant sequences reported in this paper are listed in **Table 1**.

ORIGINAL RESEARCH ARTICLE

AUTHOR CONTRIBUTIONS

W.A.H, F.B, S.A, Y.L.B, M.D.H and I.N conceived the project and analyzed the data. W.A.H performed and designed experiments. T.Y performed DNA extraction and reviewed the manuscript. T.E, J.T.B, A.L.R, F.T, J.S and M.D.H provided patients' samples and recorded the clinical data. W.A.H, F.B, M.D.H and I.N wrote the manuscript.

DISCLOSURE

The authors declare no conflict of interest.

REFERENCES

1. Sheridan C. Intrauterine growth restriction—diagnosis and management. *Aust Fam Physician*. 2005;34:717–723.
2. Azzi S, Salem J, Thibaud N, et al. A prospective study validating a clinical scoring system and demonstrating phenotypical-genotypical correlations in Silver-Russell syndrome. *J Med Genet*. 2015;52:446–453.
3. Dias RP, Nightingale P, Hardy C, et al. Comparison of the clinical scoring systems in Silver-Russell syndrome and development of modified diagnostic criteria to guide molecular genetic testing. *J Med Genet*. 2013;50:635–639.
4. Bartholdi D, Krajewska-Walasek M, Ounap K, et al. Epigenetic mutations of the imprinted IGF2-H19 domain in Silver-Russell syndrome (SRS): results from a large cohort of patients with SRS and SRS-like phenotypes. *J Med Genet*. 2009;46:192–197.
5. Netchine I, Rossignol S, Dufourg MN, et al. 11p15 imprinting center region 1 loss of methylation is a common and specific cause of typical Russell-Silver syndrome: clinical scoring system and epigenetic-phenotypic correlations. *J Clin Endocrinol Metab*. 2007;92:3148–3154.
6. Wakeling EL, Brioude F, Lokulo-Sodipe O, et al. Diagnosis and management of Silver-Russell syndrome: first international consensus statement. *Nat Rev Endocrinol*. 2016;13:105–124.
7. Gicquel C, Rossignol S, Cabrol S, et al. Epimutation of the telomeric imprinting center region on chromosome 11p15 in Silver-Russell syndrome. *Nat Genet*. 2005;37:1003–1007.
8. Azzi S, Blaise A, Steunou V, et al. Complex tissue-specific epigenotypes in Russell-Silver Syndrome associated with 11p15 ICR1 hypomethylation. *Hum Mutat*. 2014;35:1211–1220.
9. Brioude F, Oliver-Petit I, Blaise A, et al. CDKN1C mutation affecting the PCNA-binding domain as a cause of familial Russell Silver syndrome. *J Med Genet*. 2013;50:823–830.
10. Begemann M, Zirn B, Santen G, et al. Paternally inherited IGF2 mutation and growth restriction. *N Engl J Med*. 2015;373:349–356.
11. Kas K, Voz ML, Roijer E, et al. Promoter swapping between the genes for a novel zinc finger protein and beta-catenin in pleiomorphic adenomas with t(3;8)(p21;q12) translocations. *Nat Genet*. 1997;15:170–174.
12. Astrom A, D'Amore ES, Sainati L, et al. Evidence of involvement of the PLAG1 gene in lipoblastomas. *Int J Oncol*. 2000;16:1107–1110.
13. Zatkova A, Rouillard JM, Hartmann W, et al. Amplification and over-expression of the IGF2 regulator PLAG1 in hepatoblastoma. *Genes Chromosomes Cancer*. 2004;39:126–137.
14. Voz ML, Agten NS, Van de Ven WJ, Kas K. PLAG1, the main translocation target in pleomorphic adenoma of the salivary glands, is a positive regulator of IGF-II. *Cancer Res*. 2000;60:106–113.
15. Hensen K, Van Valckenborgh IC, Kas K, Van de Ven WJ, Voz ML. The tumorigenic diversity of the three PLAG family members is associated with different DNA binding capacities. *Cancer Res*. 2002;62:1510–1517.
16. Declercq J, Van Dyck F, Braem CV, et al. Salivary gland tumors in transgenic mice with targeted PLAG1 proto-oncogene overexpression. *Cancer Res*. 2005;65:4544–4553.
17. Hensen K, Braem C, Declercq J, et al. Targeted disruption of the murine Plag1 proto-oncogene causes growth retardation and reduced fertility. *Dev Growth Differ*. 2004;46:459–470.
18. Klemke M, Muller MH, Wosniok W, et al. Correlated expression of HMGA2 and PLAG1 in thyroid tumors, uterine leiomyomas and experimental models. *PLoS One*. 2014;9:e88126.
19. Zhou X, Benson KF, Ashar HR, Chada K. Mutation responsible for the mouse pygmy phenotype in the developmentally regulated factor HMGI-C. *Nature*. 1995;376:771–774.
20. De Crescenzo A, Citro V, Freschi A, et al. A splicing mutation of the HMGA2 gene is associated with Silver-Russell syndrome phenotype. *J Hum Genet*. 2015;60:287–293.
21. Fokstuen S, Kotzot D. Chromosomal rearrangements in patients with clinical features of Silver-Russell syndrome. *Am J Med Genet A*. 2014;164A:1595–1605.
22. Okada Y, Kamatani Y, Takahashi A, et al. A genome-wide association study in 19 633 Japanese subjects identified LHX3-QSOX2 and IGF1 as adult height loci. *Hum Mol Genet*. 2010;19:2303–2312.
23. Cho YS, Go MJ, Kim YJ, et al. A large-scale genome-wide association study of Asian populations uncovers genetic factors influencing eight quantitative traits. *Nat Genet*. 2009;41:527–534.
24. Gudbjartsson DF, Walters GB, Thorleifsson G, et al. Many sequence variants affecting diversity of adult human height. *Nat Genet*. 2008;40:609–615.
25. Weedon MN, Lettre G, Freathy RM, et al. A common variant of HMGA2 is associated with adult and childhood height in the general population. *Nat Genet*. 2007;39:1245–1250.
26. Chinuck R, Dewar J, Baldwin DR, Hendron E. Appetite stimulants for people with cystic fibrosis. *Cochrane Database Syst Rev*. 2014: CD008190.
27. Chao W, D'Amore PA. IGF2: epigenetic regulation and role in development and disease. *Cytokine Growth Factor Rev*. 2008;19:111–120.
28. Abi Habib W, Brioude F, Azzi S, et al. 11p15 ICR1 partial deletions associated with IGF2/H19 DMR hypomethylation and Silver-Russell syndrome. *Hum Mutat*. 2016;38:105–111.

29. Abi Habib W, Azzi S, Brioude F, et al. Extensive investigation of the IGF2/H19 imprinting control region reveals novel OCT4/SOX2 binding site defects associated with specific methylation patterns in Beckwith-Wiedemann syndrome. *Hum Mol Genet*. 2014;23:5763–5773.

30. Azzi S, Abi Habib W, Netchine I. Beckwith-Wiedemann and Russell-Silver Syndromes: from new molecular insights to the comprehension of imprinting regulation. *Curr Opin Endocrinol Diabetes Obes*. 2014;21:30–38.

31. Mott R, Yuan W, Kaisaki P, et al. The architecture of parent-of-origin effects in mice. *Cell*. 2014;156:332–342.

32. Juma AR, Damdimopoulou PE, Grommen SV, Van de Ven WJ, De Groef B. Emerging role of PLAG1 as a regulator of growth and reproduction. *J Endocrinol*. 2016;228:R45–56.

33. DeChiara TM, Efstratiadis A, Robertson EJ. A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. *Nature*. 1990;345:78–80.

34. Akhtar M, Holmgren C, Gondor A, et al. Cell type and context-specific function of PLAG1 for IGF2 P3 promoter activity. *Int J Oncol*. 2012;41:1959–1966.

35. Li X, Nong Z, Ekstrom C, et al. Disrupted IGF2 promoter control by silencing of promoter P1 in human hepatocellular carcinoma. *Cancer Res*. 1997;57:2048–2054.

36. Ekstrom TJ, Cui H, Li X, Ohlsson R. Promoter-specific IGF2 imprinting status and its plasticity during human liver development. *Development*. 1995;121:309–316.

37. Gnirke A, Melnikov A, Maguire J, et al. Solution hybrid selection with ultra-long oligonucleotides for massively parallel targeted sequencing. *Nat Biotechnol*. 2009;27:182–189.