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**11p15 ICR1 partial deletions associated with *IGF2/H19* DMR hypomethylation
and Silver-Russell Syndrome**

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Abstract

The 11p15 region harbors the *IGF2/H19* imprinted domain, implicated in fetal and postnatal growth. Silver-Russell syndrome (SRS) is characterized by fetal and postnatal growth failure, and is caused principally by hypomethylation of the 11p15 imprinting control region 1 (ICR1). However, the mechanisms leading to ICR1 hypomethylation remain unknown. Maternally inherited genetic defects affecting the ICR1 domain have been associated with ICR1 hypermethylation and Beckwith-Wiedemann Syndrome (an overgrowth syndrome, the clinical and molecular mirror of SRS), and paternal deletions of *IGF2* enhancers have been detected in four SRS patients. However, no paternal deletions of ICR1 have ever been associated with hypomethylation of the *IGF2/H19* domain in SRS. We screened for new genetic defects within the ICR1 in a cohort of 234 SRS patients with hypomethylated *IGF2/H19* domain. We report deletions close to the boundaries of ICR1 on the paternal allele in one familial and two sporadic cases of SRS with ICR1 hypomethylation. These deletions are associated with hypomethylation of the remaining CBS, and decreased *IGF2* expression. These results suggest that these

regions are most likely required to maintain methylation after fertilization. We estimate these anomalies to occur in about 1% of SRS cases with ICR1 hypomethylation.

Key Words : *IGF2/H19* imprinted domain, imprinting control region 1, deletions, hypomethylation, Silver-Russell Syndrome.

Introduction

The 11p15 telomeric domain contains imprinted genes: *IGF2* (a major fetal growth factor) expressed only from the paternal allele during fetal life and *H19* (a long non-coding RNA) expressed only from the maternal allele (Azzi, et al., 2014; Gicquel, et al., 2005). The monoallelic expression of *IGF2* and *H19* is regulated by an intergenic differentially methylated region (IG DMR) called the *H19/IGF2:IG-DMR* or imprinting control region 1 (ICR1), which is methylated on the paternal allele (Azzi, et al., 2014). ICR1 is a tandem of two blocks of repeats (A and B repeats) containing binding sites for several *trans*-acting factors, such as CTCF (binding to the unmethylated CpG and implicated in the insulator function of ICR1), ZFP57 (involved in the establishment and maintenance of DNA methylation) and pluripotency factors, such as OCT4 and SOX2 (which protect the maternal allele against *de novo* methylation during early embryogenesis) (Abi Habib, et al., 2014; Demars, et al., 2010) (Figure 1). Finally, several cohesin domains have been reported to be associated with CTCF binding sites (CBS) within and around ICR1 (Weth and Renkawitz, 2011). The difference in the CpG methylation status of the CBS of the two alleles (according to the parental origin of the allele) in cohesin complexes leads to the formation of different chromatin

loops, ultimately determining whether the genes of this domain are expressed or repressed (Nativio, et al., 2009) .

Silver-Russell syndrome (SRS, OMIM #180860) is a rare growth retardation condition associating intrauterine and postnatal growth retardation with relative macrocephaly at birth, a prominent forehead, failure to thrive during early childhood, and body asymmetry with hemihypotrophy (Azzi, et al., 2015).

The molecular defects underlying SRS are mostly epigenetic: 40 to 50% of SRS patients display hypomethylation of the *H19/IGF2*:IG-DMR/ICR1 locus, leading to low levels of *IGF2* expression and the biallelic expression of *H19* (Gicquel, et al., 2005; Netchine, et al., 2007). Another major cause is maternal uniparental disomy (mUPD) of chromosome 7 (5-10%) (Eggermann, et al., 2015). Some rare genetic mechanisms, resulting in familial cases of SRS, are also known: paternal *IGF2* loss- of-function mutation (Begemann, et al., 2015), maternal *CDKN1C* (a cell cycle inhibitor gene located in the centromeric 11p15 domain) gain-of-function mutation (Brioude, et al., 20013) and maternal 11p15 duplications (Azzi, et al., 2014). Maternal UPD and hypomethylation of chromosome 14q32 with hypomethylation of its imprinted *DLK1/GTL2* domain (Kagami, et al., 2015), and mUPD of chromosomes 16 and 20 have also been identified in patients fulfilling the clinical diagnosis of SRS according to Netchine-Harbison Clinical Scoring System(Azzi, et al., 2015).

Four SRS cases from three unrelated families with paternal deletions and rearrangements of the *IGF2/H19* enhancer regions crucial for the normal expression of these genes have been identified. None of these deletions encompassed the repeated blocks of ICR1, and only one also removed the *H19* gene and extended up

to the seventh CTCF binding site of ICR1 (which is located outside the repeated blocks) (Gronskov, et al., 2011).

Beckwith-Wiedemann Syndrome (BWS, OMIM #130650) is an overgrowth syndrome corresponding to the molecular and clinical mirror of SRS. Overall 5 to 10% of BWS patients display hypermethylation of the maternal ICR1. About 20% of these patients carry genetic defects within the maternal ICR1: large deletions encompassing several CBS, or point mutations/small deletions within binding sites for pluripotency factors OCT4/SOX2 (Abi Habib, et al., 2014; Demars, et al., 2010). These defects do not yield a pathologic phenotype if inherited paternally.

No such molecular defects have yet been found in SRS patients. We therefore searched for defects in other regions of ICR1 that might cause hypomethylation of the domain when paternally transmitted. For this purpose, we investigated ICR1 in a cohort of 234 SRS patients with ICR1 hypomethylation.

Materials and methods

Population studied

Patients were followed at Armand Trousseau Children's Hospital or were referred by other clinical centers for the molecular analysis of suspected SRS. A geneticist and/or a pediatric endocrinologist examined each patient and a detailed clinical form was completed.

Blood samples were sent to the same molecular diagnosis laboratory, at Trousseau Hospital, in all cases. We included 234 SRS patients with demonstrated 11p15

H19/IGF2:IG-DMR/ICR1 hypomethylation in this study. All patients displayed hypomethylation of the *H19/IGF2:IG-DMR/ICR1* CBS2 [median methylation index (MI) 22%, range: 0-44%; normal range: 46-56%] and *H19DMR* [median methylation index (MI) 21%, range: 0-45%; normal range: 46-56%]. These patients had a normal methylation pattern for *KCNQ1OT1* TSS DMR/ICR2 (median MI 50%, range: 46-54%; normal range: 41-54%). MI was determined by ASMM real-time qPCR on DNA extracted from blood leukocytes (Azzi, et al., 2011).

Ethical considerations: written informed consent for participation was received for all patients, either from the patients themselves or from their parents, in accordance with French national ethics rules for patients recruited in France (Assistance Publique–Hôpitaux de Paris authorization no. 681) and the IRB I00000204 at the Mount Sinai School of Medicine, NYC, USA for patients recruited in the United States of America.

Nucleic acid extraction and quantification

DNA was extracted from blood leukocytes, by an in-house protocol, after cell lysis by a salting-out procedure, as previously described (Abi Habib, et al., 2014). RNA was extracted from skin fibroblasts with the NucleoSpin miRNA kit for the isolation of small and large RNAs (Macherey-Nagel, France).

DNA and RNA were quantified with a NanoDrop ND-1000 spectrophotometer (Invitrogen, France).

Culture of fibroblasts from skin

Fibroblasts isolated from the skin of a patient carrying the 2.6 kb deletion, five SRS patients and five sex and age matched control fibroblast samples provided by Coreill

Cell Repositories (New Jersey, USA) were cultured to confluence in RPMI 1640 (Gibco, Cergy Pontoise, France) supplemented with 10% fetal calf serum and 50 U/ml-50 µg/ml ampicillin/streptomycin at 37°C. The cells were detached by trypsin treatment and centrifuged. The cell pellets obtained were washed twice with 1x PBS and then used for the extraction of DNA and RNA.

Bisulfite treatment of DNA

Genomic DNA (1 µg) was treated with sodium bisulfite, with the EZ DNA Methylation kit (Zymo Research, Orange, CA), in accordance with the manufacturer's instructions. Genomic DNA was eluted in 50 µl RNase-free H₂O and stored at -20°C.

TaqMan allele-specific methylated multiplex real-time quantitative PCR (ASMM real-time qPCR) and methylation analysis

The methylation status of the imprinted loci of the 11p15 region (*KCNQ1OT1* TSS DMR/ICR2, *IGF2*DMR0, *H19/IGF2*:IG-DMR/ICR1 CBS1, CBS2, CBS3, CBS4, CBS5, CBS6, CBS7 and *H19*DMR) was assessed by ASMM real-time qPCR, as previously described (Azzi, et al., 2011). The methylation index (MI) at each locus was determined by calculating the ratio of methylated to unmethylated alleles as follows: (number of methylated alleles/total number of methylated and unmethylated alleles) x 100. The ASMM real-time qPCR primer and probe sequences are listed in supplementary table S1.

Multiplex ligation-dependent probe amplification

We used 100 ng of genomic DNA for the multiplex ligation-dependent probe amplification (MLPA) analysis. MLPA was performed with the ME030-C3 BWS/RSS kit from MRC-Holland (Amsterdam, The Netherlands), in accordance with the

manufacturer's instructions. MLPA data were analyzed with Coffalyser.NET software from MRC-Holland.

SNP microarray analysis

Large copy number variations, parental origin of copy number variation and uniparental disomy were investigated on Illumina CytoSNP-12 arrays (Illumina, San Diego, CA, USA). More accurate detection of the 135 kb deletion breakpoints was performed using the SurePrint G3 Unrestricted Human CGH Microarray ISCA v2, 4x180K from Agilent (Agilent, Santa Clara CA).

Sequencing of the ICR1 A1/B1 and B6/B7 repeats

The ICR1 A1/B1 and B6/B7 repeats, containing the CBS6 and CBS1 flanking regions, respectively, were sequenced for 210 patients by standard Sanger sequencing methods, with the ABI PRISM Big Dye Terminator v3.0 Cycle Sequencing Kit and an ABI 3100 genetic analyzer, as previously described (Abi Habib, et al., 2014). The sequencing products were then analyzed with SeqScape v2.6 (Life Technologies, Courtaboeuf, France). We did not sequence these regions for the remaining 24 patients for whom MLPA was performed, because too little DNA was available for these patients.

Reverse transcription and real-time PCR quantification of mRNAs

We synthesized cDNA from 1.5 µg of total large RNAs from fibroblasts with the SuperScript II Reverse Transcriptase system, in accordance with the manufacturer's instructions (Life Technologies, France). The cDNA was then diluted to a final volume of 200 µl. Gene expression (3 µl of diluted cDNA per well) was quantified with the Power SYBR Green Kit (Life Technologies, France) on an ABI-7900HT (Life

Technologies, France). The amount of each transcript present was normalized against β -actin (a housekeeping gene stably expressed in both patients and controls). Expression levels in controls were arbitrarily set to 1 and fold changes (FC) between the two groups were calculated as $FC = 2^{-\Delta\Delta C_t}$. The primer sequences used were: β -Actin-F: AGAGCTACGAGCTGCCTGAC, β -Actin-R: GCACTGTGTTGGCGTACAG (NM_001101.3), *IGF2*-F: TGGACACCCTCCAGTTCGTC, *IGF2*-R: GCGGAAACAGCACTCCTCAA (NM_000612.5).

Results

Genetic defects affecting ICR1

Multiplex ligation-dependent probe amplification (MLPA) revealed three large deletions, two within ICR1 and the third encompassing ICR1 and the 3' end of the *IGF2* gene (Figures 1-2, table 1).

One of the deletions within ICR1 was found in a familial case, in an SRS patient with ICR1 hypomethylation who inherited the deletion from his affected father (IV-1 and III-2, respectively, in Figure 3). The SRS phenotype was also reported in the half-brother (IV-3) of patient IV-1 and their paternal uncle (III-5), but no DNA samples from these individuals were available for analysis in our laboratory. The other two deletions occurred *de novo* on the paternal allele, as shown by SNP microarray analysis and ICR1 sequencing (Figure 2). SNP array and subsequent array-CGH

allowed us to describe a high mosaicism level for the large sporadic case 1 deletion affecting about 90% of cells.

We estimate the prevalence of these genetic defects at about 1% of SRS patients with ICR1 hypomethylation.

The available clinical data for the two patients carrying the *de novo* deletions and for patient IV-1 are reported in table 2.

Newly identified deletions in ICR1

We compared these newly identified deletions with those previously reported to affect ICR1 associated with BWS and known not to cause SRS when transmitted paternally. We identified two new regions close to the boundaries of ICR1 that were deleted in the three SRS patients and were not among the deletions reported for BWS (Figure 1). The first of these regions was located within the A1/B1 repeat; it harbored the sixth CTCF binding site (CBS6), and was deleted in the patient with familial SRS. The second region was located within the B6/B7 repeat; it included the first CTCF binding site (CBS1), and was deleted in both *de novo* cases. The sequencing of these two regions in 210 other SRS patients revealed no other deletions, but several unrecorded single-nucleotide changes were found in seven patients (Table 3). We were unable to determine the parental transmission pattern for these variants, due to a lack of parental DNA.

DNA methylation profile of the *IGF2/H19* domain in patients with ICR1 deletions

We determined the methylation levels of all the CBS within ICR1, and of *H19*DMR and *IGF2*DMR0 (two secondary DMRs located in the promoters of *H19* and *IGF2*,

respectively; Figure 1). As expected, no methylation was observed at the deleted CBSs (because of the lack of the normally methylated paternal allele). The remaining CBSs were completely unmethylated in the two sporadic cases, but partially methylated in the familial case. Hypomethylation of the secondary DMRs was also detected in all patients. All methylation indices were similar in the affected father and son (Figure 3, patients III-2 and IV-1, respectively).

Expression of the *IGF2* gene

We finally compared cultured skin fibroblasts *IGF2* relative expression from sporadic case 2 carrying the 2.6 kb deletion and 5 SRS patients with complete hypomethylation of the *IGF2/H19* domain (without genetic defects within the ICR1) to age and passages number matched controls. Both sporadic case 2 and SRS patients' fibroblasts barely expressed *IGF2* (Figure 4).

Discussion

We report deletions within the paternal allele for the 11p15 ICR1 in a large cohort of SRS patients with hypomethylation of the *IGF2/H19* domain. None of the maternal deletions within ICR1 previously described in BWS patients resulted in a pathological phenotype on paternal transmission (Abi Habib, et al., 2014; Azzi, et al., 2014; Begemann, et al., 2012; Beygo, et al., 2013). Only paternal deletions of the enhancers common to *IGF2* and *H19* were implicated in SRS without methylation defects of the ICR1 (Gronskov, et al., 2011). The deletions described in this study occur in a region close to the boundaries of the ICR1 that has never been implicated in the disease, whereas deletions within ICR1, but away from the boundaries, have been reported in BWS. Several studies in mouse models have shown that i)

deletions of the four CTCF binding sites within the endogenous paternal DMD (homologous to the human ICR1) have no effect on the imprinting status of the domain despite abnormal levels of methylation (Engel, et al., 2006), whereas ii) large deletions within the DMD and/or its 5' segment (a CpG-rich region without CTCF binding sites), which are methylated in sperm, lead to a partial decrease in DMD methylation after fertilization (Fedoriw, et al., 2004; Matsuzaki, et al., 2015; Thorvaldsen, et al., 1998; Thorvaldsen, et al., 2002). The deletion of the 5' segment decrease *Igf2* expression and increase *H19* expression when transmitted paternally, leading to lower body weight for mutant embryos (Matsuzaki, et al., 2015; Thorvaldsen, et al., 1998) (as reported for *Igf2*-knockout mice (DeChiara, et al., 1990)). These experiments demonstrate that the 5' segment of the murine DMD is essential for the maintenance of its imprinting status after fertilization and during early embryogenesis. The syntenic region of the human ICR1 was found to be deleted in two sporadic cases from our SRS cohort with hypomethylation of the entire telomeric domain. This deletion led to decreased expression of *IGF2* consistent with the phenotype and the physiological function of ICR1. Another deletion in the 3' segment of human ICR1 was found in a familial case. This deletion caused SRS when paternally transmitted. We were unable to check the level of ICR1 methylation in the sperm of the familial SRS case. However, we hypothesize that these two segments at the boundaries of the human ICR1 are involved in maintaining methylation between fertilization and blastocyst stage, rather than establishing methylation in sperm as reported in mice (Ginart, et al., 2016; Matsuzaki, et al., 2015). A recent study on mouse embryonic fibroblasts from a mouse model of SRS with mutations in the paternal ICR (Engel, et al., 2004) showed heterogeneity in methylation and mono/biallelic expression (Ginart, et al., 2016). This

would explain why patient IV-1, carrying the familial deletion, and sporadic case 2 have slight body asymmetry despite the germline nature of the deletion, that can be explained by an epigenetic mosaicism at the level of the individual cell in accordance with findings in the mouse model (Ginart, et al., 2016). Moreover, the body asymmetry of case 1 (whose deletion removes the last 3 exons of *IGF2*) may also be due to the mosaic state of the somatic deletion, occurring at a postzygotic stage.

The relative position of the deletions (5' or 3') affected methylation in different ways. Indeed, deletions of the ICR1 5' segment led to more severe hypomethylation of the remaining CBSs than 3' deletions. The methylation indices for the remaining CBS and both the *IGF2* and *H19* DMRs were identical in the affected father and son. These findings suggest that i) each deletion has a different effect on methylation maintenance, probably by altering different mechanisms and ii) hypomethylation occurs in a well-defined, non-stochastic manner. However, we cannot exclude the possibility of common regulative elements present on both fragments, with more severe effect when deleted in the 5' segment of the ICR1.

We have also observed that hypomethylation was not equally spread between the remaining CBS. Such heterogeneity in methylation indices has been observed upon maternal deletions in BWS patients, where hypermethylation occurs differently at each CBS of the ICR1. While, in those BWS patients, the hypermethylation depends on the distance between the CBS and the affected OCT4 binding sites, the mechanisms leading to the hypomethylation in SRS patients with paternal deletions reported here are yet to be deciphered (Abi Habib, et al., 2014; Beygo, et al., 2013).

Several single-nucleotide polymorphisms were identified within these two newly identified regions, but they were considered unlikely to be involved in

hypomethylation, because i) several SNPs near these variants have already been described and ii) no binding sites or motifs were found at these sites in data from the ENCODE project or *in silico* predictions.

Regarding genetic counseling, both the 2.2 kb (familial case) and 2.6 kb (sporadic case 2) deletions could lead to BWS upon maternal transmission, because they overlap with the OCT4 binding site within the A2 repeat known to protect the maternal ICR1 from hypermethylation (Abi Habib, et al., 2014). Upon paternal transmission, these deletions could lead to a recurrent SRS phenotype.

In conclusion, we report here the first deletions of ICR1 associated with hypomethylation of the *IGF2/H19* domain leading to SRS. ICR1 deletions account for about 1% of SRS cases with ICR1 hypomethylation and their detection would be useful to help guide genetic counseling. These deletions close to the boundaries of the ICR1 suggest that these regions are required for methylation maintenance after fertilization. Further studies will be required to determine the dynamics of ICR1 methylation maintenance during development.

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Conflict of Interest statement. None declared

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Figure Legends

Figure 1: Schematic representation of the *H19/IGF2:IG-DMR/ICR1* and the new paternal deletions described in this study. Black solid lines represent previously reported deletions at the boundaries of the ICR1 in patients with BWS upon maternal transmission and normal phenotype upon paternal transmission. Methylation indices (%) are shown above each CBS and DMR.

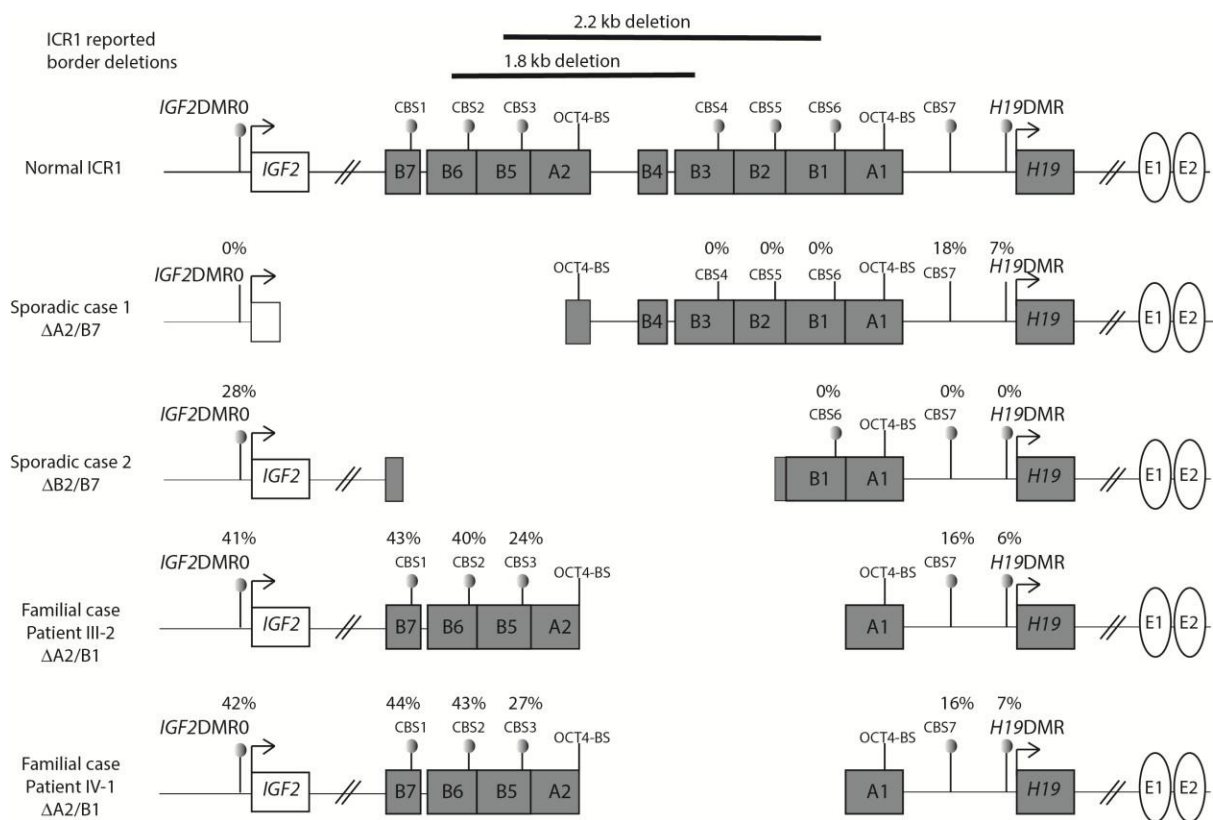


Figure 2: Detection of the deletions by MLPA and determination of their paternal origin using SNP array for sporadic case 1 and Sanger sequencing of both normal and deleted ICR1 for sporadic case 2.

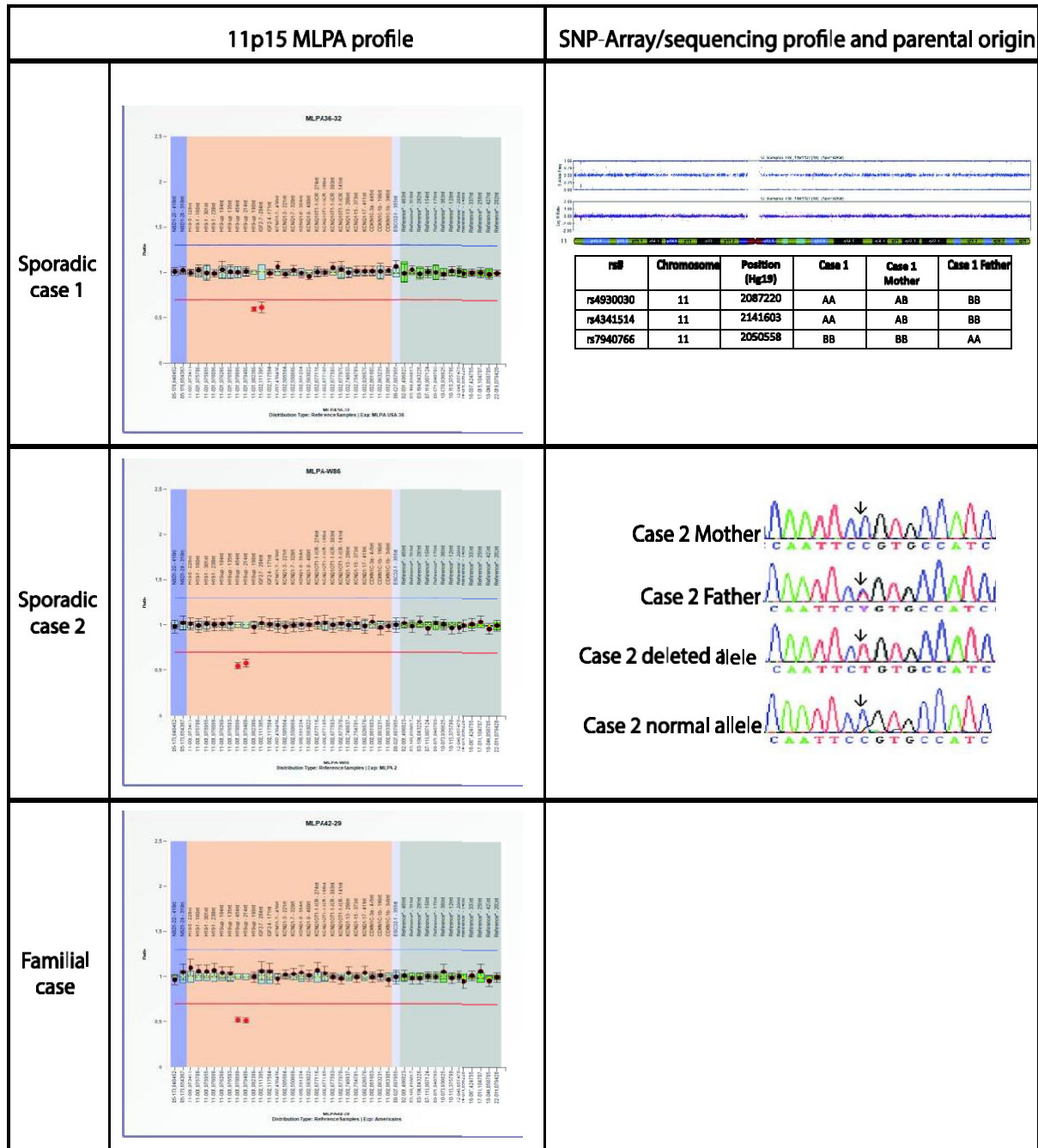


Figure 3: Pedigree of the familial case, showing SRS transmission upon paternal inheritance of the 2.2 kb deletion within the *H19/IGF2:IG-DMR/ICR1*. DEL+: deletion detected, DEL-: Deletion not detected, N.A: Not available for testing. II.1 is considered to be a very likely carrier of the deletion, despite the lack of DNA for testing.

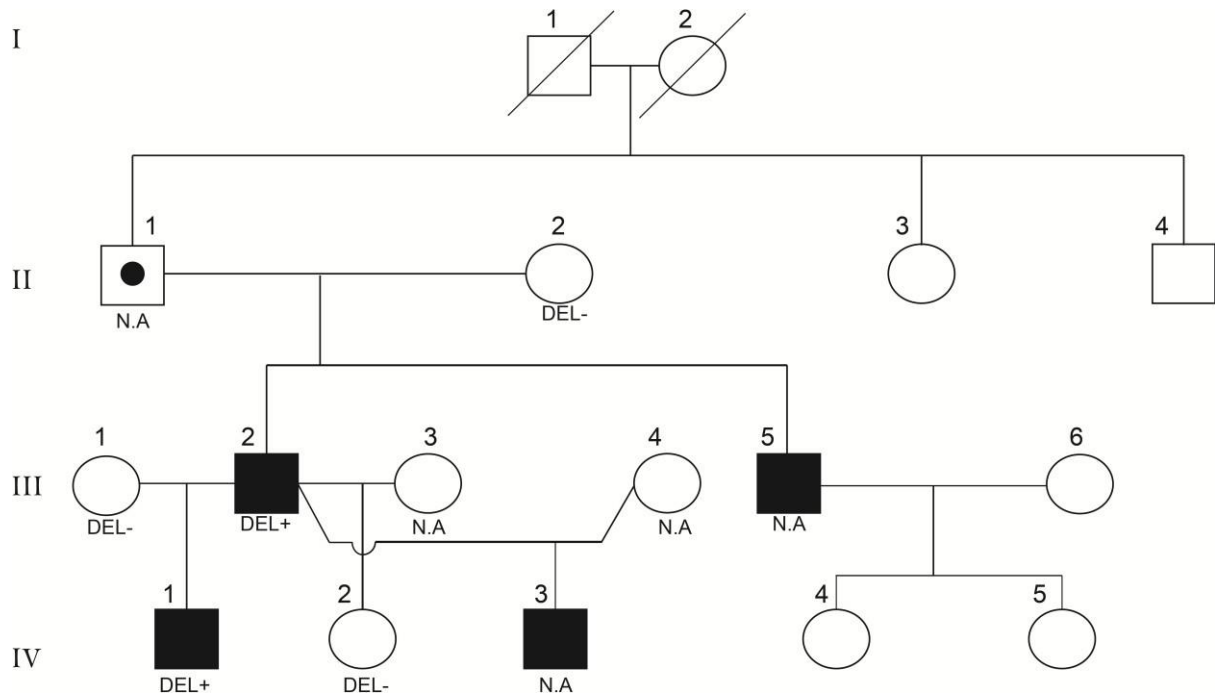
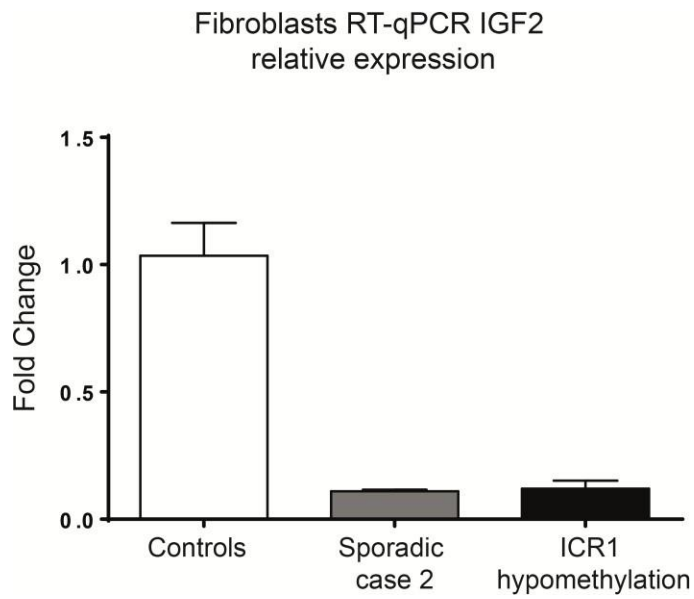


Figure 4: Expression levels of *IGF2* in fibroblasts from the sporadic case 2 and 5 SRS patients with complete hypomethylation of the *IGF2/H19* domain compared to controls.



Familial/sporadic	Transmission	Parental allele	Position (Hg19)	Size	Affected domains	Deleted IGF2 exons (NM_001007139)
Sporadic case 1	<i>de novo</i>	Paternal	chr11:2023132-2158149	135 Kb	CBS1-CBS2-CBS3	3-4-5
Sporadic case 2	<i>de novo</i>	Paternal	chr11:2021577-2024221	2.6 Kb	CBS1-CBS2-CBS3-CBS4-CBS5	
Familial	Germline	Paternal	chr11:2020760-2023010	2.2 Kb	CBS4-CBS5-CBS6	

Table 1: Characteristics of the ICR1 deletions found in SRS patients.

	Sporadic case 1	Sporadic case 2	Familial Case IV-1
Sex	Female	Male	Male
Term at birth (weeks of gestation)	38.57	32	36
Birth length cm/SDS	41.91/-4.55	30.5/-7	40.6/-5.56
Birth weight g/SDS	1620/-3.96	890/-3.7	2270/-2.62
Birth head circumference cm/SDS	33.4/0.88	29/-0.43	35.9/0.76
Height at 2 years cm/SDS	73.8/-3.45	78/-4.1	71.9/-4.21
BMI at 2 years /SDS	14.1/-4.65	7.9/-6	15.9/-0.4
Head circumference at 2 years cm/SDS	47.7/0.5	ND	ND
Relative macrocephaly at birth	Yes	Yes	Yes
Prominent forehead during infancy	Yes	Yes	Yes
Feeding difficulties during infancy	Yes	Yes	No
Body asymmetry	Yes	Mild	Mild
N-H SRS scoring system	6/6	6/6	5/6
Growth hormone therapy	Yes, at age of 2.5 years	Yes	Yes, at age of 2 years

Table 2: Clinical presentation in the patients studied. Gray boxes show the criteria for SRS according to (Azzi, et al., 2015); BMI below -2 SDS at the age of 2 years and feeding difficulties were considered as a unique positive criteria. Relative macrocephaly is defined as a difference of at least +1.5 SDS between head circumference and body length/weight at birth. ND: not determined

Variation	Position (Hg19)	Domain
C>T	chr11:2,023,521	B6/B7 repeat
C>G	chr11:2,023,742	B6/B7 repeat
C>T	chr11:2,023,956	B6/B7 repeat
G>A	chr11:2,024,213	B6/B7 repeat
G>C	chr11:2,020,538	A1/B1 repeat
C>T	chr11:2,021,459	A1/B1 repeat
C>T	chr11:2,021,910	A1/B1 repeat

Table 3: Sequence variations of the ICR1 A1/B1 and B6/B7 regions found in SRS patients.