

# **Title: New Developments in Silver-Russell Syndrome and Implications for Clinical Practice**

## **Keywords**

Silver-Russell Syndrome, Genomic imprinting, Epigenetics, Fetal growth, Epigenomic Editing, Discordant twins, Multilocus Imprinting disturbance (MLID)

## **Summary**

Silver-Russell syndrome (SRS) is a clinically and genetically heterogeneous disorder, characterized by pre- and postnatal growth restriction, relative macrocephaly, body asymmetry and characteristic facial features. It is one of the imprinting disorders, which results as a consequence of aberrant imprinted gene expressions. Currently, maternal uniparental disomy of chromosome 7 (upd(7)mat) accounts for approximately 10% of SRS cases, while ~50% of patients have hypomethylation at imprinting control region 1 (ICR1) at chromosome 11p15.5 locus, leaving ~40% of cases with unknown etiologies. This review aims to provide a comprehensive list of molecular defects in SRS reported to date, and to highlight the importance of multiple-loci/tissue testing and trio (both parents and proband) screening. The epigenetic and phenotypic overlaps with other imprinting disorders will also be discussed.

## **Silver-Russell syndrome**

Silver-Russell syndrome (SRS) [OMIM ID: 180860] is an imprinting disorder affecting pre- and postnatal growth. To date, more than 400 cases have been reported since its first description by Silver et al in 1953 and Russell in 1954 who independently reported a subset of children with low birth weight, short stature, body asymmetry and characteristic facial features [1, 2].

Genomic imprinting is an epigenetic modification process, which allows a gene to be expressed in a monoallelic, parent-of-origin specific manner. This phenomenon which is almost exclusive to the placental mammals, was identified in the 1980s through a set of mouse experiments [3, 4]. Since then more than 100 imprinted genes have been identified in mice and approximately half of these are conserved in humans (<http://www.har.mrc.ac.uk/>). The imprinted expression is regulated by differential methylation of imprinting control regions (ICRs) which are established in germline during gametogenesis [5]. Disturbance in the expression dosage of imprinted genes due to uniparental disomy (UPD), epi/mutation and copy number variation (CNV) could lead to imprinting disorders, mainly characterised by growth, metabolic and neurological abnormalities [6]. There are currently 10 reported imprinting disorders in humans: Angelman syndrome [OMIM 105830], Prader-Willi syndrome [OMIM 176270], Transient neonatal diabetes mellitus 1 [OMIM 601410], Pseudohypoparathyroidism Type Ib [OMIM 603233], Silver-Russell syndrome, Beckwith-Wiedemann Syndrome [OMIM 130650], Temple syndrome [OMIM 616222], Kagami-Ogata syndrome [OMIM 608149], Maternal uniparental disomy of chromosome 20 syndrome and Precocious puberty syndrome (<http://www.imprinting-disorders.eu/>).

Diagnosis of SRS is relatively difficult as it has many clinical features (summarised in Table 1). To date, six clinical scoring systems of SRS have been suggested [7-12]. Majority of them diagnose SRS by the presence of three or four out of the following characteristics: birth weight  $\leq$  -2SD, postnatal growth restriction, relative macrocephaly, facial characteristics and body asymmetry (Table 1). Accurate diagnosis is a challenge since

postnatal growth measurements requires follow-up, and facial features become less obvious with age. The most recently described “Netchine-Harbison clinical scoring system include feeding difficulties in addition to the aforementioned features, and having four out of these six features diagnose “Likely-SRS” [7]. Screening 69 patients with this system identified 98% of the SRS patients with SRS-associated molecular anomalies. It is interesting to note that, in their “Likely-SRS double negative” group which have no ICR1 hypomethylation or upd(7)mat, one patient was found to have upd(20)mat. It is a molecular defect found in patients with upd(20)mat syndrome, a newly defined imprinting disorder characterised by pre/post-natal growth restriction and feeding difficulties [13]. Another patient had hypomethylation at *DLK1/MEG3* intergenic DMR (IG-DMR), which is a characteristic of Temple syndrome (TS). TS is another imprinting disorder sharing clinical features with SRS. In addition, a recent review by Fokstuen and Kotzot pointed out that none of the systems include delayed bone age, even though it is observed in most of the upd(7)mat patients [14]. Therefore, a consensus in diagnostic criteria of SRS would be useful, and a European-wide consensus is due to be published in 2016 (personal information).

## **Molecular anomalies of SRS**

Majority of SRS cases are sporadic, but various modes of inheritance including recessive, dominant and X-linked have been suggested [15]. To date, numerous chromosomal aberrations have been associated with SRS, including Chromosome 1, 2, 7, 8, 11, 12, 13, 14, 15, 16, 17, 18, 20, 21, 22 and X (summarised in Suppl. Table 1), with majority of anomalies attributed to chromosome 7 and 11 (Fig. 1). One has to be cautious that some of these reported cases include SRS-like patients who might not fit the definitive criteria.

### ***Chromosome 7***

Cytogenetic abnormalities of chromosome 7 have been reported in several SRS cases (Suppl. Table 1; Fig. 2). UPD refers to the inheritance of two copies of a chromosome pair from a single parent, where heterodisomy is the inheritance of both parental homologues (grandparental pair) and isodisomy is the transmission of two identical homologue of one parent. Maternal UPD for chromosome 7 (upd(7)mat) occurs in approximately 10% of SRS cases [16]. Chromosome 7 was first linked to SRS aetiology through identification of a cystic fibrosis patient with SRS-like features [17], followed by reports of growth restricted upd(7)mat patients born to *CFTR* heterozygous mothers but carried homozygous mutations, resulted from isodisomy for chromosome 7 [18, 19]. Therefore, exposure of recessive mutations has been previously suggested as the cause of SRS in upd(7)mat patients. However, no common isodisomic region on chromosome 7 was identified in five SRS cases investigated [20], making it unlikely to be the causative mechanism for SRS. The same study also indicated that most SRS upd(7)mat are likely to arise from meiosis I nondisjunction error, followed by trisomic rescue as evident by heterodisomy at the centromeres. This is in contrast to the BWS cases with paternal UPD11 upd(11)pat where all reported cases are isodisomic resulting in upd(11)pat and normal cell mosaic, implying post-zygotic mitotic nondisjunction error [21]. Trisomy 7 mosaicism as a consequence of trisomy rescue, has also been ruled out as the possible mechanism for SRS, since no trisomic cells have been detected in upd(7)mat patients' lymphocytes or fibroblasts [22], and subjects with confined placental mosaicism for trisomy 7 have normal birth weight [23].

It is therefore most likely that the pathogenesis of SRS with upd(7)mat is attributed to the disrupted expression of imprinted genes on chromosome 7. Reduced expression of growth promoting paternal gene(s) or overexpression of maternally expressed gene(s) which has growth limiting function could lead to the disorder. Importantly, the four reported cases with paternal isodisomy for chromosome 7 (upd(7)pat) do not exhibit growth and developmental phenotypes, except for one patient who became overweight at 3 months of age, and another who showed growth restriction at 6 months of age but this was likely to be owing to his recessive disease conditions [24-27]. Therefore, rather than the loss of paternally expressed growth-

promoting genes, overexpression of growth-limiting maternal genes on chromosome 7 may be more relevant for SRS growth restriction phenotype. Although there is a SRS-like case with paternal deletion of Chr7q32, including the paternally expressed gene mesoderm specific transcript (*MEST*) [28].

The candidate SRS regions within chromosome 7 were narrowed down following identification of SRS patients with maternal segmental duplications affecting 7p11.2-p13 locus and maternal segmental UPD involving 7q31-qter region [29-35]. The 7p11.2-p13 region encompasses an imprinted growth factor receptor bound protein 10 gene (*GRB10*), and biallelically expressed growth related genes (*IGFBP1*, *IGFBP3* and *EGFR*). The 7q31-qter region harbours four imprinted genes (*CPA4*, *MEST*, *COPG2* [conflicting reports] and *KLF4*) and two imprinted non-coding RNAs (*MESTIT1* and *COPG2IT1*). However, screening the coding regions of these candidate genes have so far failed to identify pathogenic mutations although it should be noted that some of these patients might have carried ICR1 hypomethylation [36]. Though, one study reported a patient with 7p11.2-p12 maternal duplication without SRS phenotypes and the duplication did not include *GRB10*, lending the possibility of involvement of this gene in SRS pathogenesis [32]. In addition, a recent report presented a case with *GRB10* hypermethylation with an additional Chr20p13 microdeletion [37].

It is possible that there are more imprinted genes that have not been discovered yet within this region. Recently, Hannula *et al* [38] searched for novel DMRs and imprinted genes on chromosome 7 by comparing nine upd(7)mat, one upd(7)pat and 10 control whole blood DNA samples using the Infinium 450K HumanMethylation array. Two of the identified DMRs mapped to the previously predicted imprinted genes *HOXA4* (7p15.2) and *GLI3* (7p13), and one at *SVOPL* gene (7q34), and monoallelic expression of *HOXA4* and maternal expression of *SVOPL* were confirmed in blood. A recent large-scale study assessing allele-specific expressions in 1,678 human RNA-seq samples from 45 tissues in 178 individuals, also revealed interesting results [39]. *DDC* gene adjacent to *GRB10*, which was previously shown to be imprinted in mice and biallelically expressed in multiple human fetal tissues [40], was shown to be imprinted in multiple adult human tissues including brains. Another disputed imprinted gene, *DLX5* which maps to the *PEG10/SGCE* imprinted cluster at 7q21.3, was shown to be biallelically expressed in mice but imprinted in humans. The observed discrepancies could possibly be explained by the difference in the types and developmental stages of tissues used. In addition, some genes were missed in their study due to their stringent inclusion criteria such as requiring four expressed heterozygous SNPs per gene for parent-of-origin analysis. Interestingly, the same study also identified the mouse *Copg2* to be biparentally imprinted, a phenomenon previously reported for *Igf2* and *Grb10* [41]. Although it was not assessed in human samples, *Copg2* showed paternal expression in E15 brain and maternal expression in E17.5 brain, adding further complexity. In conclusion, the roles of imprinted genes on chromosome 7 in SRS aetiology require further investigation.

### **Chromosome 11p15.5**

The imprinted cluster at chromosome 11p15.5 contains two independent regulatory domains: the telomeric *IGF2/H19* domain controlled by the paternally methylated ICR1 and the centromeric *KCNQ1* domain controlled by the maternally methylated ICR2 (also known as KvDMR1) (Fig. 3). Both ICR1 and ICR2 acquire differential methylation in the germline, which resets in each generation to lay sex specific imprints during gametogenesis [5].

Hypomethylation of the ICR1 is observed in 31-63% of SRS cases (Suppl. Table 1). The ICR1 is located 2-4kb upstream of *H19*, and it controls the reciprocal imprinted expression of the paternally expressed growth promoter *IGF2* and maternally expressed growth repressing non-coding RNA gene *H19* [42]. There are seven binding sites for the zinc finger protein CTCF (CCTC-binding factor) at ICR1. The binding of the

CTCF protein to the unmethylated maternal ICR1 blocks the *IGF2* promoter from interacting with the shared enhancers downstream of *H19*, resulting in maternal *H19* expression. The protein cannot bind to the paternally methylated ICR1 which allows the paternal *IGF2* expression (Fig. 3). Therefore, hypomethylation of ICR1 is thought to cause suppression and de-repression of paternal *IGF2* and *H19*, respectively, by permitting the CTCF protein to bind to the paternal ICR1, leading to a growth restriction phenotype [43]. Strikingly, hypermethylation at ICR1 and hypomethylation of ICR2 is observed in approximately 5% and 50% of Beckwith-Wiedemann syndrome (BWS) patients respectively, which is an overgrowth syndrome suggested to be phenotypically and genotypically opposite to SRS.

SRS patients have been shown to carry variable ICR1 methylation levels in different tissues. This epigenetically mosaic pattern strongly indicates post-zygotic epigenetic modification error, which is manifested as body asymmetry phenotype observed in most patients. Further evidence comes from twin studies. To date, nine discordant monozygotic SRS twins and only one concordant twin have been reported [43-52]. Notably, discordant BWS MZ twin cases are predominantly females with the reported male to female ratio of 1:12 [53]. Similarly, discordant SRS MZ twins show a modest skew with male to female ratio of 2:7. Investigations of the ICR1 methylation levels have been carried out on three of the nine discordant MZ twins so far. Two of the twin pairs showed ICR1 hypomethylation in leucocyte DNA of both the affected and unaffected pair but in other tissues such as buccal cells and skin fibroblast, only the affected individual showed hypomethylation for the ICR1 [43, 45]. In the third discordant MZ twin however, hypomethylation was detected only in the affected twin's blood [52]. This discrepancy could be attributed to the presence or absence of shared circulation in the placenta. All cases were monochorionic-diamniotic twins, 70% of which is estimated to share blood circulation [54]. This would result in transferring of hypomethylated hematopoietic stem cells (HSCs) from the affected to the unaffected twin, resulting in similar methylation levels in the leucocyte. The third case showed no obvious shared circulation in the placenta, consistent with the distinguished methylation pattern between the twins in their blood cells [52].

### ***DNA methylation and mRNA expression***

ICR1 hypomethylation is assumed to lead to a decrease in the overall *IGF2* expression and increase in *H19*. Several studies have tested the correlation between the ICR1 methylation level and *IGF2* or *H19* expression levels in different tissues. Gicquel *et al* showed that the *IGF2* expression level is lower in the affected discordant twin's fibroblast, and that *H19* was biallelically expressed, indicating loss of imprinting [43]. More recently, Azzi *et al* reported that ICR1 hypomethylation levels in fibroblasts correlated with the *IGF2* expression, but when compared to controls, the difference was not significant [55]. Allelic expression of the *H19* was not assessed in these patients and the *H19* level did not correlate with the degree of ICR1 methylation. The same study also assessed the tissue specific epigenotypes, including blood, fibroblasts, right and left buccal cells of the patients with semi-hypertrophy, showing variable levels of the methylation. Eleven percent of the patients showed discordance between leukocytes and buccal cells, strongly suggestive of post-zygotic epigenetic variation leading to mosaic epigenetic profiles. On a protein level, ICR1 hypomethylation does not cause reduced IGF-II serum level, although this might not represent its function in prenatal period and in other tissues [56].

### ***MLID and SRS***

A subset of patients with imprinting disorders has been found to carry more than one DNA methylation abnormality, termed multilocus imprinting disturbance (MLID) [57]. Of the 10 imprinting disorders described, five them have been reported with MLID: 50-75% of transient neonatal diabetes mellitus type 1 (TNDM1) patients with *PLAGL1/HYMAI* DMR hypomethylation, 4.5-50% of Pseudohypoparathyroidism type Ib (PHP-Ib) with epimutation at one of the Chr20q13 DMRs, 20-45% of BWS with ICR2

hypomethylation, 7-36% SRS with ICR1 hypomethylation and a single case with Prader-Willi Syndrome (PWS)/BWS features (Summarised in [58, 59]). The wide frequency range of MLID in these patients may reflect the differences in the sample sizes, ethnicities, techniques, clinical scoring systems and number of loci investigated. Unlike the TNDM1 MLID cases, SRS and BWS patients with MLID are not significantly clinically distinguished from patients with isolated epimutations at ICR1 and ICR2, although an increased developmental delay in the MLID groups was reported [60].

Strikingly, SRS and BWS cases with nearly identical epimutations have been reported [45, 51, 60, 61]. Azzi *et al* identified three SRS and one BWS carrying both ICR1 and ICR2 hypomethylation [61]. Moreover, one of these SRS patients and the BWS case also had *PLAGL1* DMR hypomethylation despite the absence of TNDM1 phenotypes, adding further complexity to the molecular aetiology. From these studies it is estimated that approximately 4% of ICR1 hypomethylated SRS cases also have additional ICR2 hypomethylation. Of note, ICR2 hypomethylation in some of these cases were restricted to leucocytes but absent in their buccal cells, showing evidence of epigenetic mosaicism [60]. Interestingly, ICR2 hypomethylation has previously been identified in growth restricted cases [51, 62], and in clinically normal ART-conceived individuals [63]. Since ICR2 hypomethylation has been considered a hallmark for the overgrowth disorder BWS, the reason for such shared epimutations in phenotypically normal or opposite disorders is currently unknown. It has been suggested that there is an ‘epidominance’ effects, where the phenotype is dominated by the most severely affected loci [55]. Also, since the majority of the study is carried out in blood, the mosaic nature of epimutation makes it difficult to determine the degree to which the most functionally relevant tissue is affected.

Despite the shared epimutations identified in SRS and BWS, additional epimutations at the Chr14q32 imprinting locus is so far predominant in SRS MLID patients. Hypomethylation of paternally methylated IG-DMR and *MEG3*-DMR at *DLK1/MEG3* loci on Chr14q32 is associated with Temple Syndrome (TS) (previously referred to as upd(14)mat syndrome) [64]. TS shares characteristic features with other imprinting disorders. Pre/post-natal growth restriction, hypotonia and small hands are observed in both TS and PWS [65]. Pre/post-natal growth restriction, intellectual disability, speech delay are seen in approximately equal frequency amongst SRS and TS patients. Other symptoms such as body asymmetry, relative macrocephaly, prominent forehead, fifth finger clinodactyly, feeding difficulties, hypoglycaemia, ear abnormalities and micrognathia are described in both syndromes but at higher frequency in SRS. Body asymmetry especially was suggested to be a useful SRS indicator, whereas precocious puberty and obesity are specific to TS. TS patients also have different characteristic facies from SRS, such as broad forehead, short nose with wide nasal tip and relatively short philtrum [64]. There are four SRS cases reported either having isolated 14q32 epi/mutations or in combination with upd(7)mat (Suppl. Table 1). These patients exhibit either SRS phenotypes only or characteristics for both SRS and TS. On the other hand, MLID SRS patients with both ICR1 hypomethylation and Chr14q32 epimutation generally have typical SRS phenotypes [61]. Although these cases constitute a small proportion of SRS it does suggest that an additional test for Chr14q32, especially in atypical or idiopathic cases is worth considering.

### ***Fetal genetic causes of the epigenetic errors***

Genetic causes for MLID is being explored and mutations in several *trans*-acting factors have been reported in MLID patients and their mothers [66-69]. Homozygous or compound heterozygous mutations within a *trans*-acting *ZFP57* gene have been shown to be associated with hypomethylation at multiple imprinted loci in approximately 5% of TNDM1 patients [68]. *ZFP57* encodes a Krüppel-associated box domain (KRAB) zinc finger protein which has been shown to bind to most germline imprinted DMRs (iDMRs) in a parental allele-specific manner, and is required for DNA methylation maintenance at these loci in early embryonic stages [70]. Despite this, screening of 30 SRS patients with ICR1 hypomethylation and three SRS with

MLID did not reveal any pathogenic mutation within *ZFP57*, therefore the involvement of this gene in the aetiology of SRS is currently unknown [45, 71].

### ***Maternal effect genes***

Another mechanism for MLID has been suggested through maternal-effect mutations. Three members of the NLRP family have been associated with imprinting disorders and aberrant DNA methylation. A single case of homozygous frameshift mutation in *NLRP2* was reported in a mother with two BWS children and ICR2 hypomethylation in a consanguineous family [69]. Homozygous or compound heterozygous mutations in *NLRP7* and *KHDC3L* have been identified in women who had recurrent biparental hydatidiform moles, with evidence of multiple iDMR-specific methylation defects [66, 72]. More recently, maternal rare variants in *NLRP5* have been associated with MLID and imprinting disorders [67]. They have screened 39 MLID cases (SRS, BWS, TNDM and undiagnosed cases) and 33 mothers identifying five mothers carrying mutations, two of whom had SRS children. One mother was a compound heterozygote (c.1664G>T; c.2320T>C) and had atypical SRS child with cleft lip and palate, had a BWS child with a different partner and six miscarriages in total. The second mother was heterozygous for c.1156\_1158dupCCT, and had discordant monozygotic SRS twins. The authors suggest that the biallelic mutation (compound heterozygous) may have more severe effects than monoallelic mutations (heterozygous), although one mother was homozygous for a mutation (c.1699A>G) and had three healthy children. Since the mutations occur in heterozygous, compound heterozygous and homozygous forms, it is difficult to assess the functional model, but possibly suggesting a dominant negative or near loss-of-function mutations manifested by variable phenotypic outcome.

### ***Familial SRS involving Chr11 gene mutations***

Mutations in two imprinted genes at Chr11p15.5, cyclin-dependent kinase inhibitor 1C (*CDKN1C*) and *IGF2*, have recently been reported in familial SRS cases [73, 74]. *CDKN1C* is a preferentially maternally expressed tumour suppressor gene under the control of ICR2 (Fig. 3), and is mutated in approximately 10% of BWS [75]. Most BWS cases are sporadic but approximately 15% are familial, 40% of which carry *CDKN1C* mutations. *CDKN1C* protein has three domains: a cyclin-dependent kinase inhibitory domain (CKI), a proline and alanine (PAPA) repeat domain and a QT domain which contains a proliferating cell nuclear antigen (PCNA) binding domain [76]. The BWS-*CDKN1C* pathogenic variants are maternally transmitted dominant loss-of-function missense mutations clustered to the CKI domain or truncation mutations spread across the gene [77].

Remarkably, gain-of-function mutations in *CDKN1C* have been identified in IMAGE syndrome (intrauterine growth restriction, metaphyseal dysplasia, congenital adrenal hypoplasia, and genital anomalies) patients, who carried missense mutations localised to the PCNA-binding domain [76]. Since *CDKN1C* is thought to act as the growth suppressor, it is likely that loss-of-function leads to overgrowth (BWS) while gain-of-function results in growth restriction (IMAGE). Moreover, Brioude, *et al* reported that screening 97 idiopathic SRS cases identified a missense mutation (c.836G>T) in a family with nine affected SRS members, six of whom was tested and shown to carry maternally inherited mutation [74]. The c.836G>T mutation is also within the PCNA domain, and at the same amino acid position as one of the reported IMAGE patients with different amino acid substitutions (IMAGE: p.Arg279Pro and SRS: Arg279Leu). The authors suggested that the increased stability of the mutant protein implies gain-of-function mutation. No further mutations were identified in the rest of 91 cases. More recently, paternally inherited *IGF2* nonsense mutation (c.191C>A) was reported in a family with four members affected with pre/post-natal growth failure with SRS-like phenotype, possibly suggesting the role of *IGF2* in post-natal growth [73]. This is an

interesting observation since *IGF2* is mainly produced in the liver after birth where it is expressed biallelically [11], with its main role is generally considered to be at prenatal growth period.

### ***CNV and structural variation at Chr11***

A number of copy number variants (CNVs) at 11p15.5 imprinting cluster have been reported in SRS patients, affecting approximately 1 % of all SRS cases (Suppl. Table 1). There are two paternally expressed and six maternally expressed imprinted genes under the control of ICR1 and ICR2, and the majority of CNVs involve maternal duplication of both ICR1 and ICR2, which is anticipated to result in excess of maternally expressed growth suppressor genes (Fig. 3). One of the interesting observations is that maternal duplication of the whole *H19/IGF2* domain including *IGF2* enhancer, *H19*, ICR1 and *IGF2* does not cause SRS but maternal duplication of the *H19/IGF2* domain excluding the *IGF2* gene does result in SRS [78], possibly indicating that doubling the *H19* expression may be sufficient to cause SRS.

## **Molecular Diagnostics and Investigations for SRS**

### ***Current diagnostics process***

For the molecular diagnosis of SRS patients, the most thorough test would be to carry out the trio whole genome bisulfite/sequencing (WGS and WGBS) to simultaneously identify UPDs including upd(7)mat, ICR1 hypomethylation and MLID, *de novo* and inherited CNV, genomic mutations in maternal (e.g. *NLRPs*) and fetal (e.g. *CDKN1C*, *IGF2*) candidate genes and any novel epi/mutations. However, it is currently not a cost effective method and the data analysis is still not hands-on for the routine clinical diagnostics settings. A suggested diagnostic flow path is shown in figure 4. In many diagnostics laboratories, methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) is used routinely. This allows simultaneous detection of methylation levels at ICR1 and ICR2, as well as CNV at 11p15.5 in a single experiment. The resulting CNV could be further confirmed by microsatellite typing or qPCR. For those who are ICR1 hypomethylation negative, microsatellite analysis (MSA) for upd(7)mat test is also typically performed. Together these would identify approximately 60% of established molecular anomalies of SRS. The advantage and disadvantages of the currently available techniques for the diagnostics and for research studies are summarized in the table 2.

### ***Molecular investigations for research studies***

The importance of multilocus testing in imprinting disorders is becoming more apparent. Therefore, it is preferred that the initial methylation testing include several imprinting DMRs. We and others have recently used high density methylation array to search genome-wide for methylation defects in SRS patients [79-82]. Interestingly, by using Illumina's 450K Infinium HumanMethylation array to look for epimutations in the idiopathic SRS patients, we have identified two SRS patients previously shown to carry normal ICR1 levels by restriction digestion method testing a single CpG, to have moderate ICR1 hypomethylation [81]. This suggests an advantage of using different and more sensitive techniques for the DNA methylation quantification and also having multiple probes at the target loci. To overcome the heterogeneous nature of SRS, we have used a round-robin statistical method so that the control methylation levels are compared to individual SRS patients rather than comparing against the SRS as a group. One problem with the 450K array, as well as with the newer 850K MethylationEPIC BeadChip, is that they do not have probes targeting at Chr14 *DLK1/MEG3* IG-DMR loci, which is implicated in the TS as discussed earlier. Therefore, if 450K/850K EPIC array were used, this region has to be investigated by an alternative method.

The majority of methylation analyses use conventional bisulfite conversion method, which means that it will not be able to distinguish 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC). This would result in over-estimation of the methylation levels. Ten-eleven translocation (Tet) enzyme can convert 5mC to

5hmC, which is thought to be an intermediate of the active demethylation step [83]. Its distribution profile in the imprinted regions, and possible correlation with imprinting disorders remains to be addressed. Recently, Matsubara *et al* reported low levels of 5hmC at IG-DMR and *MEG3*-DMR in peripheral blood of Kagami-Ogata syndrome (KOS14) patients who have hypermethylation at these loci, concluding that the 5hmC has only minor contribution to the increased methylation [84]. Also, higher 5hmC abundance was observed in their neural samples, consistent with the previous observation that 5hmC is enriched in the brain tissues and in embryonic stem cells [85]. Further characterisation of 5hmC profile in other imprinting disorders will increase the understanding of the molecular pathology.

## **Current treatment practice**

Due to the heterogeneous nature of the SRS phenotypes, treatments from different clinical specialists may be required according to their specific symptoms. These include clinical geneticists for clinical and molecular diagnosis alongside paediatric endocrinologists for observation of growth trajectory and decision on growth hormone administration. The multi-specialist team also need to include dieticians and gastroenterologists for feeding problems, paediatric surgeons if intervention required for the body asymmetry, dental specialists, speech therapists, psychologists and clinical genetic counsellors for advice on family planning.

### ***Feeding therapy***

The majority of SRS patients exhibit feeding difficulties in early childhood, seen as lack of appetite, food fussiness and slow eating [86]. Gastrointestinal complications are common in SRS children, including gastroesophageal reflux, oesophagitis and food aversion [87, 88]. These unpleasant experiences are likely to be contributing factors for their reduced appetite. It is important to monitor and maintain the adequate caloric intake for the patients in order to prevent associated problems such as hypoglycaemia, and to promote their growth. A recent study reported that malnutrition affects 70% of SRS children, and suggested considerations for providing enriched diet, enteral feeding and nutritional supplements for improvement of nutritional status [88]. Nevertheless, the feeding difficulties have been reported to gradually improve with age, and around 6 years of age, food refusal and fussiness are not significantly different from normal children [86].

### ***Growth hormone treatment***

Children born SGA (Small for Gestational Age) typically experience catch-up growth during the first year of life which completes around 2 years of age [89]. This is not generally the case for the SRS patients; a study of 386 SRS patients showed that mean adult height was 151.2 cm in males and 139.9 cm in females [90]. Recombinant growth hormone (GH) therapy is available for SGA patients including SRS, who have not demonstrated adequate catch-up growth by age two, which was approved by US Food and Drug Administration (FDA) in 2001 [91]. In Europe, the use of GH was officially indicated by European Medicines Agency in 2003 (formally known as European Agency for the Evaluation of Medicinal Products in 1995–2004), for children with growth failure who are  $\geq 4$  years old and have height less than  $-2.5$  SD and  $1$  SD below mid-parental height SDS. GH therapy in SRS children has shown an evidence of beneficial outcome. A long-term follow-up study of 26 SRS children treated with GH for the median of 9.8 years showed a significant increment of median height from  $-2.7$  SD at the start to  $-1.3$  SD final height [92]. Whether the effect of GH is influenced by the different molecular subtypes of SRS is currently unknown. One study reported a trend that the GH response was better in the upd(7)mat group compared to the ICR1 hypomethylation group (upd(7)mat  $n=5$  and ICR1 hypo  $n=19$ ) [93]. However, these studies are still in the preliminary stage and it should be investigated further in a larger sample size, as it would influence the

decision on whether to give or when to give GH. Nevertheless, the first focus on management of growth should focus on adequate caloric intake as GH cannot work without adequate glycogen stores.

### ***Long-term follow ups***

There is a paucity of information available on the long-term health issues with SRS patients, although a small number of reports indicate unremarkable medical problems. As a consequence, they are rarely followed up to the adulthood. The rare familial cases of SRS (e.g. *IGF2*, *CDKN1C* and Chr15p15.5 CNV) implies that SRS patients are fertile, although precise reproductive fitness of other molecular groups (ICR1 hypomethylation/ upd(7)mat/ idiopathics) are not well documented. The DNA methylation patterns are reset in every generation therefore the recurrence risk is low, unless it is caused by underlying genetic mutations (e.g. maternal effect mutation). However, Bartholdi *et al* has reported a father to daughter transmission of ICR1 hypomethylation in a single family [8]. Upd(7) mat are most likely caused by non-disjunction followed by trisomic rescue so the recurrent risk is also low [20].

In a recent follow up study, three Japanese male SRS adults in their early 20s were reported who have developed Type 2 diabetes, hypertension and obesity [94]. They were all male patients and had ICR1 hypomethylation. This finding is consistent with the frequently reported observations that low birth weight is associated with increased risk of metabolic abnormalities and cardiac diseases as adults [95]. Such observations have led to the hypothesis commonly known as the DOHaD (Developmental Origins of Health and Disease), which suggests that suboptimal intrauterine or early life environment, such as low maternal calorie intake during pregnancy, would cause long-term programming, possibly via epigenetic modifications, to have increased susceptibility to adulthood diseases. One of the ongoing studies to follow up SRS patients include the “Study of Adults and Adolescents with Russell-Silver Syndrome in the UK (STAARS UK)” to observe the long-term health issues and the effects of growth hormone. This would benefit the patients in terms of life-style choice and parental decision regarding growth hormone treatment.

### ***Looking forward-development of technology***

Recent advances in epi/genome engineering technology hold a potential for targeted epigenetic therapy in the future. Present epigenetic drugs (epi-drugs) such as DNMT inhibitors (g.a. 5-aza-2'-deoxycytidine), lead to genome-wide modification which is not desirable especially for the imprinting disorders with specific epimutations. Zinc finger nucleases (ZFNs) and transcription-activator-like effector nucleases (TALENs) systems are early methods developed for targeted *in vivo* genetic modifications, both of which are based on protein-DNA binding interaction [96]. More recently, the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas systems, based on guide RNA (gRNA)-DNA pairing, has demonstrated several advantages over the ZNF and TALEN methods in terms of simplicity of the design, efficiency and the ability to target multiple sites at once [96]. For site-specific DNA methylation modification, a catalytically inactive Cas9 (dCas9) variant, which can bind but cannot cleave DNA, could be fused to *de novo* methyltransferase DNMT3a for methylation and to TET1 enzyme for demethylation. So far, locus-specific DNA methylation modification have been successfully demonstrated by the ZFP coupled with the DNMT3a catalytic domain for methylating *Maspin* and *SOX2* gene promoters in breast cancer cells [97], and TALE-TET1 fusion protein for demethylation of endogenous gene promoters (*KLF4*, *HBB* and *RHOXF2*) in three human cell lines [98]. Further improvement on off-target effects and delivery methods are required for therapeutic use.

## **Conclusion & Future Prospective**

This review aimed to summarise the molecular defects associated with SRS to provide an up-to-date overview, and to highlight the importance of multilocus testing. Due to the heterogeneous presentation of SRS, a consensus in diagnostic criteria would be required. This problem was discussed in a recent meeting in Barcelona October 2015 amongst SRS experts, and the consensus is currently being prepared to publish in 2016 (personal communication: European Molecular Genetics Quality Network). Although ICR1 hypomethylation is observed in more than half of SRS patients, the underlying mechanisms of how the loss of methylation leads to the phenotype have not been fully explained. In addition, how the upd(7)mat and ICR1 hypomethylation could both lead to same disorder remains unsolved. Also, majority of the studies correlating ICR epimutation and gene expression levels only investigate the immediate neighbouring genes—*IGF2* and *H19* in case of SRS patients. Therefore, it would be interesting to investigate the transcriptome and proteome of ICR1 and upd(7)mat patients to elucidate the whole picture of molecular network in the growth pathway. Due to the phenotypic overlaps with other imprinting disorders and growth restriction disorders, final diagnosis of SRS should be based on both, thorough genetic and epigenetic testing, which would also assist/support the genetic counselling and family planning. Finally, follow-up studies on SRS patients could possibly improve prognosis of the patients through tailored therapy based on each individual's molecular defects.

## Executive Summary

- We need a consensus in SRS diagnosis criteria.
- More than half of SRS patients have ICR1 hypomethylation and about 10% have upd(7)mat.
- SRS patients require multilocus testing and trio screening.
- SRS epimutation shows mosaic pattern; therefore, multi-tissue testing is recommended.
- Phenotypic/epigenetic overlap with other imprinting disorders including TS and upd(20)mat, are becoming apparent.
- There is a modest skew in male to female ratio in SRS monozygotic twin with the ratio of 2:7.
- ICR1 hypomethylation group have more “classic” SRS phenotypes compared to upd(7)mat group.
- There are not significant phenotypic differences between MLID SRS and ICR1 hypomethylation, except that developmental delay was more frequent in MLID group.

## Financial disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript.

## Figure legends

**Figure 1. Summary of reported molecular anomalies of SRS.** MLID = multilocus imprinting disturbance. ICR1 = imprinting control region 1. Upd(7)mat = maternal uniparental disomy of chromosome 7.

**Figure 2. Human chromosome 7 ideogram with imprinted genes and chromosomal aberrations reported in SRS patients.** Letters (Red = maternal expression, Blue = paternal expression, purple = biparental imprinting expression and black = biallelic expression). \*The imprinting statuses of *DDC*, *DLX5* and *COPG2* in humans have conflicting results. Please refer to the online version for the colour image.

**Figure 3. Schematic representation of the human imprinted cluster at chromosome 11p15.5 implicated in SRS and BWS.** Maternal and paternal chromosomes are represented in pink and blue respectively. ICR

= imprinting control region. Paternal (blue), maternal (pink) and biallelic (black) and the direction of the transcription are indicated by arrows. Figure orientations: left = centromeric and right = telomeric. The CTCF protein can bind to the unmethylated ICR1, blocking the *IGF2* from accessing the downstream enhancers. The methylated ICR1 do not allow CTCF binding, resulting in *IGF2* promoter to interact with the enhancers. Please refer to the online version for the colour image.

#### Figure 4. Suggested SRS diagnostics flow chart.

To test for all known SRS molecular anomalies, combination of different diagnostics techniques should be performed. Blue shade indicates the techniques used in the routine diagnostics (Only widely used techniques shown here). Orange shade indicates the techniques that can be used optionally or in the research studies. MLID = multilocus imprinting disturbance. ICR1 = imprinting control region 1. CGH = comparative genomic hybridisation. NGS = next-generation sequencing. Please refer to the online version for the colour image.

## Tables

### Table 1. SRS clinical characteristics with their frequencies and diagnostics scoring system comparison.

Frequencies (%) are based on n= 64 SRS patients (n= 44 with ICR1 hypomethylation and n= 20 upd(7)mat) studied by Wakeling *et al.* [99]. To date, 6 diagnostic scoring systems have been reported. BW = birth weight. BL = birth length. SD = standard deviation. SGA = small for gestational age (defined as birth weight and/or length  $\leq -2$  SD score). \*The Netchine-Harbison clinical scoring system reported by Azzi *et al* (2015) [7] is based on the Netchine *et al* (2007) [11] scoring system, where SGA is no longer mandatory (having four out of six of the features diagnose them as “Likely-SRS”). Adapted with permission from Wakeling *et al.* [99]

### Table 2. A summary of techniques used for SRS diagnostics and research investigations.

Currently no single technique could test for all known SRS molecular anomalies. Therefore, combinations of different techniques should be performed. Blue shade indicates the techniques used in the routine diagnostics (Only widely used techniques shown here). Orange shade indicates the techniques that can be used optionally or in the research studies. ○ = suitable, Δ = partially suitable and X = not suitable. UPD = uniparental disomy, CNVs = copy number variations, MS = methylation specific, FISH = Fluorescent in situ hybridization, CGH= comparative genome hybridization, qPCR= quantitative polymerase chain reaction, MS SNUPE = Methylation-specific single nucleotide primer extension, NGS = next generation sequencing, MeDIP-seq = methylated DNA immunoprecipitation sequencing, MS-MLPA = methylation-specific multiplex ligation probe-dependent amplification. MLID = multilocus methylation disturbance. ICR1= imprinting control region 1.

## Supplementary Table

### Supplementary Table 1. Molecular findings associated with SRS.

Not all additional epimutation loci as a consequence of MLID in ICR1 hypomethylated patients are included (e.g. IG-DMR, *PEG3*, *GNAS* and *IGF2R*). Chr = chromosome. dup = duplication. del = deletion. mos = mosaic. upd= uniparental disomy. Mat = maternal. Pat = paternal. DMR = differentially methylated region. ICR = imprinting control region. r = ring chromosome. t = translocation. inv = inversion. PAR = pseudoautosomal region. TS = Temple Syndrome. DGS = DiGeorge syndrome. BWS = Beckwith-Wiedemann syndrome.

Figure 1.

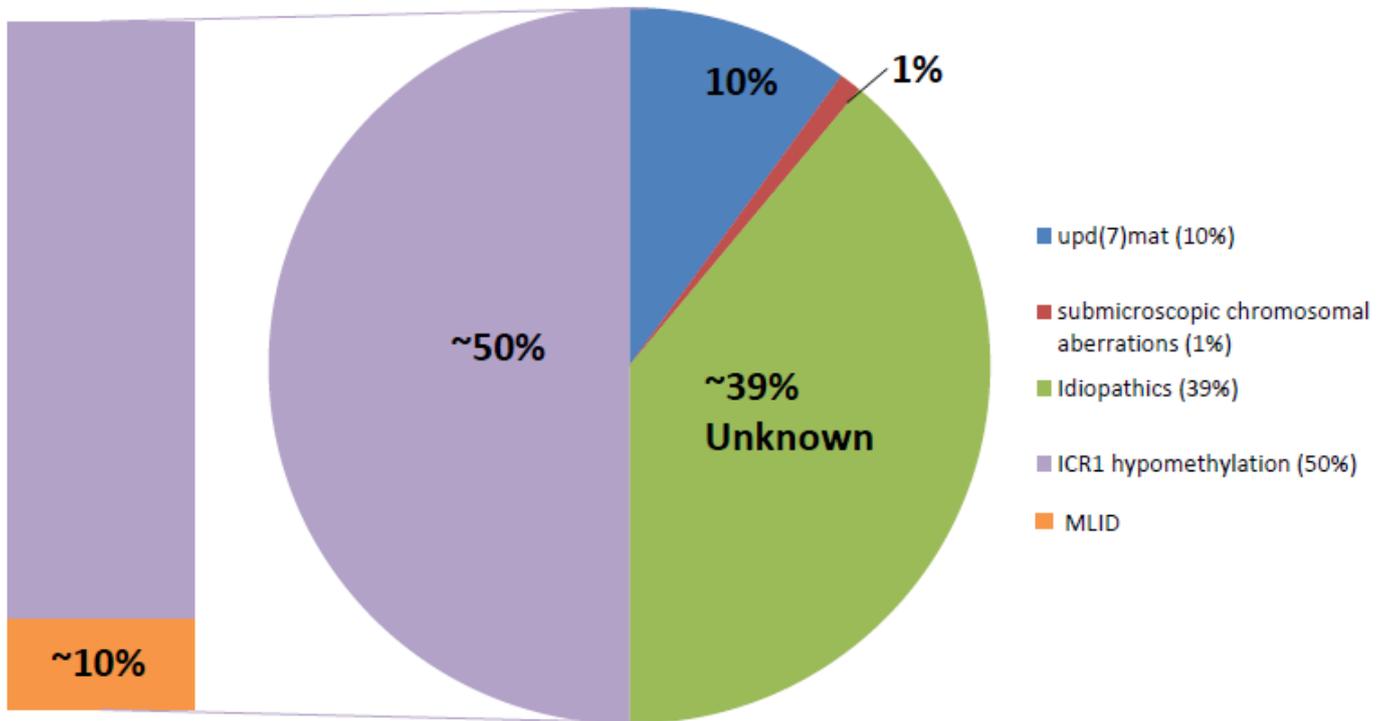


Figure 2.

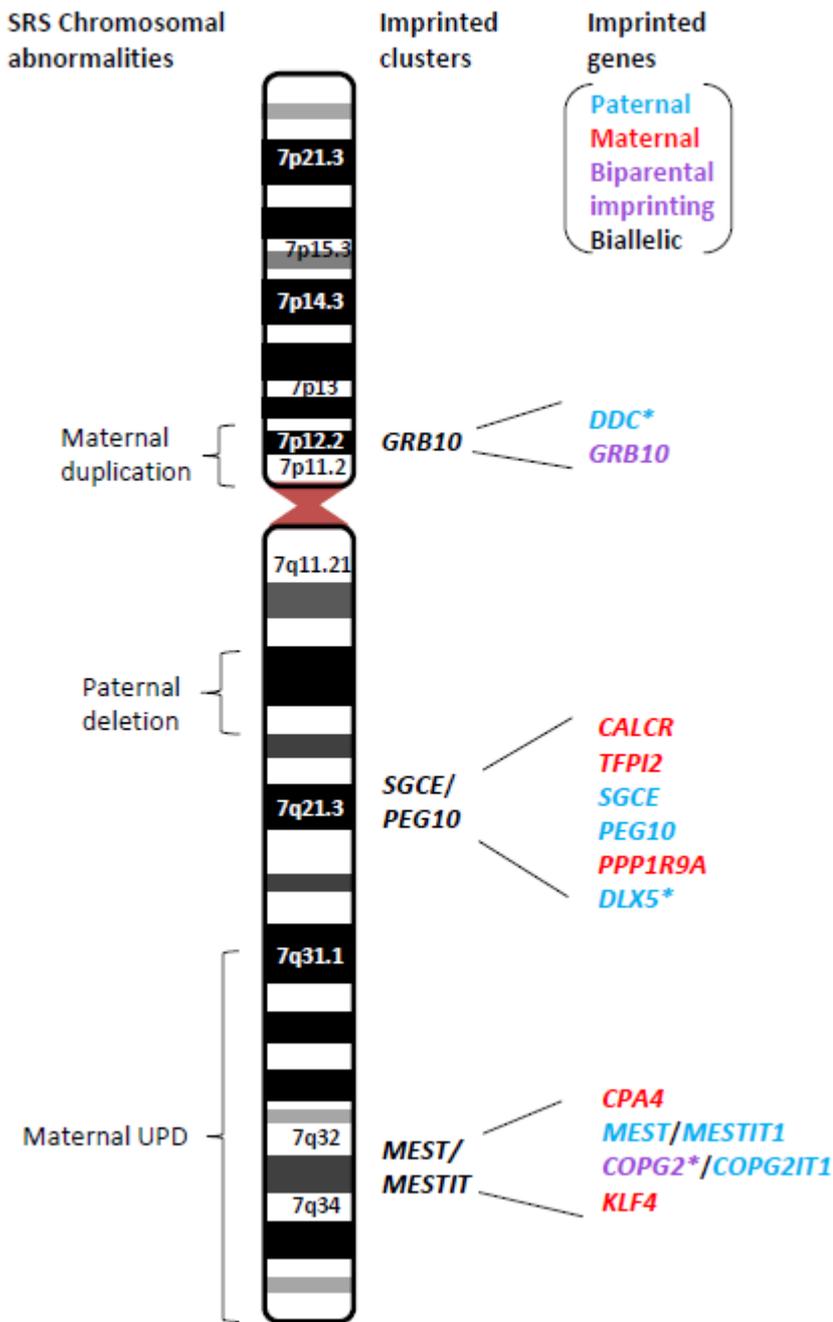


Figure 3.

SRS/BWS locus: Chr 11p15.5

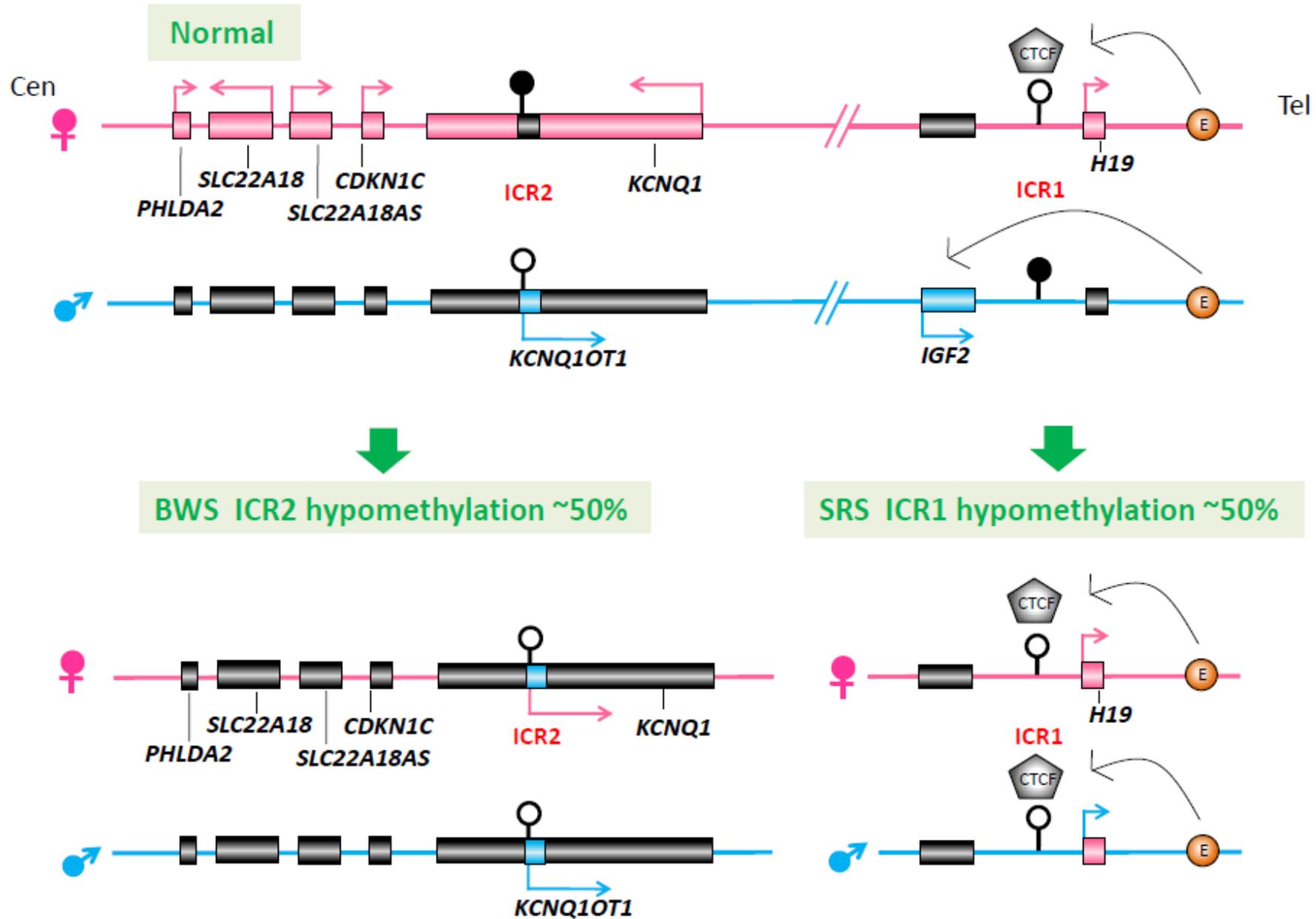
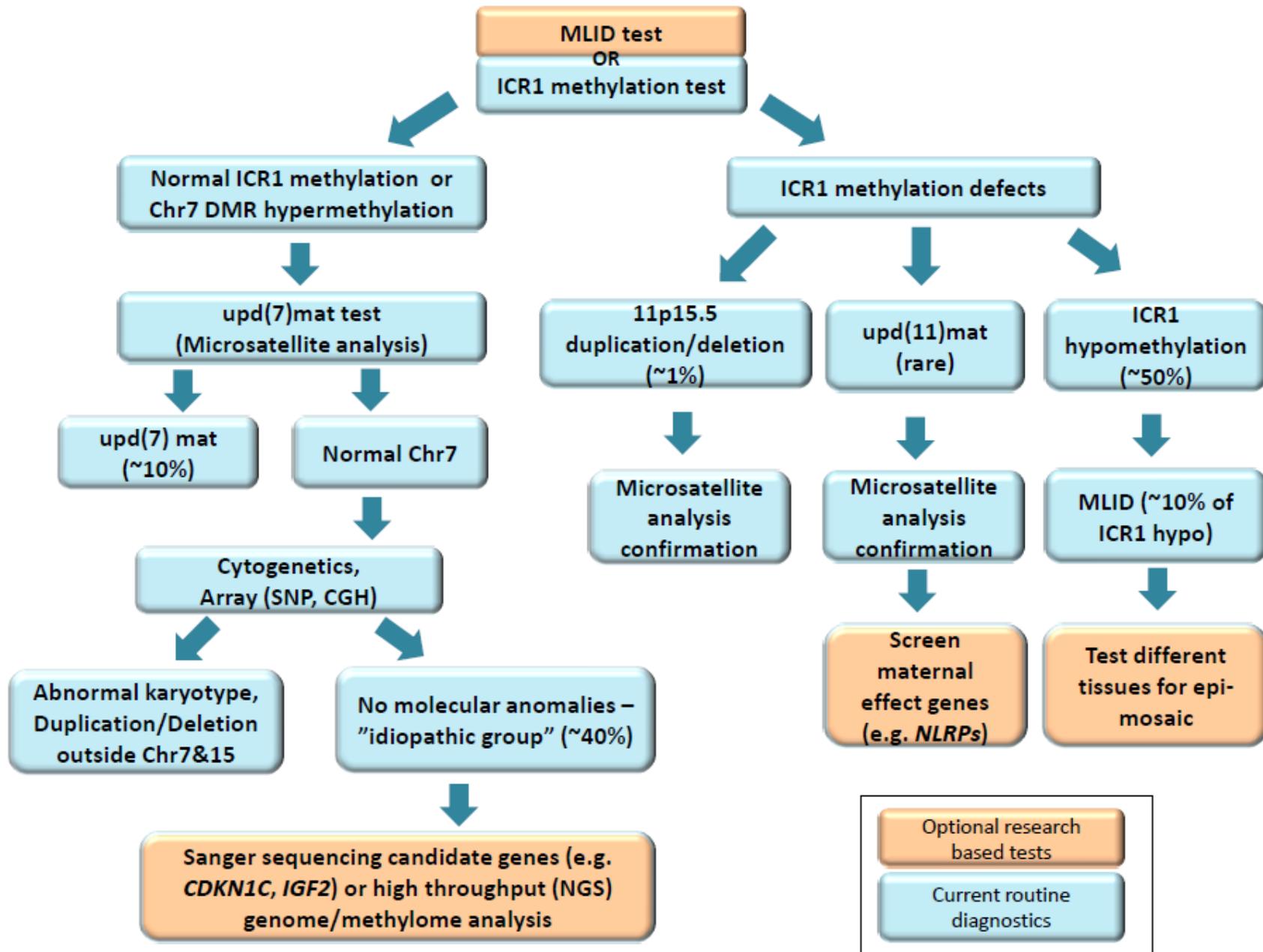


Figure 4.



**Table 1. SRS clinical characteristics with their frequencies and diagnostics scoring system comparison.**

	Molecular sub-groups		Reported diagnostics criteria for SRS					
	ICR1 hypo (n=44) (%)	upd(7)mat (n=20) (%)	Lai <i>et al</i> (1994)	Price <i>et al</i> (1999)	Netchine <i>et al</i> (2007)	Bartholdi <i>et al</i> (2009)	Dias <i>et al</i> (2013)	Azzi <i>et al</i> (2015)*
<b>Growth</b>								
Birth weight $\leq$ -2 SD	82	70	√	√	√ (SGA)	√ (BW) √ (BL) $\leq$ 10 <sup>th</sup> centile	√	√ (SGA)
Height at examination $\leq$ - 2 SD	57	65	√	√	√	√ $\leq$ 3 <sup>rd</sup> centile	√ (after 2 years)	√
Relative macrocephaly	70	90		√	√	√ √ (normal HC)	√	√
Body asymmetry	68	30	√	√	√	√ (face) √ (body) √ (limbs)	√	√
<b>Craniofacial features</b>			√	√		√ (others)		
Triangular face	59	90				√		
Frontal bossing	60	60			√	√		√
Micro/retrognathia	64	35						
Dental crowding	36	45						
Down-turned corners of the mouth	30	20						
Thin upper lip	27	30						
Low-set/posteriorly rotated ears	36	75						
<b>Developmental</b>						√ (normal)		
Global delay	20	65						
Speech delay	39	50						
<b>Other clinical signs</b>								
5th finger clinodactyly	75	45	√			√		
2/3 toe syndactyly	23	20						
Camptodactyly	16	25						
Café-au-lait macules	14	15						
Excessive sweating	64	75						
Hypoglycaemia	24 (n=37)	29 (n=17)						
Feeding difficulties	84	90			√			√
Gastro-oesophageal reflux	14	10						
Otitis media	14	20						
Delayed closure of fontanelles	43 (n=28)	36 (n=11)						
Movement disorder	0	15						
<b>Congenital abnormalities</b>						√ (others)		
Cleft palate/bifid uvula	7	0						
Congenital heart defect	9	0						
Male genital anomaly	23 (n=22)	0 (n=5)				√		
Renal anomaly	0	10						
Scoliosis	9	5						
<b>Scores required for diagnosis of SRS</b>			$\geq$ 3/5	$\geq$ 4/5	SGA + $\geq$ 3/5	$\geq$ 8/15	$\geq$ 3/4	$\geq$ 4/6

**Table 2. A summary of techniques used for SRS diagnostics and research investigations.**

	Methods	upd(7) mat (~10%)	Chromosomal aberrations	Candidate gene mutation screen	New candidate gene/region detection	ICR1 hypo (~50%)	MLID	Pros & Cons
Genomic	Microsatellite analysis	○	○	x	x	x	x	Pros: Fast and only routine molecular biology techniques required. Cons: Targets one locus at a time. Does not distinguish UPD and CNV. Could be difficult for mosaic sample.
	Cytogenetic analysis	x	○	x	Δ	x	x	Pros: Investigates genome-wide. Cons: Low resolution (>5 Mb).
	FISH	x	○	x	x	x	x	Pros: Investigates genome-wide. Cons: Low resolution. Requires prior knowledge of the affected locus for the probe design.
	Array (e.g. CGH/SNP)	○	○	x	Δ	x	x	Pros: Investigates genome-wide in high resolution. Cons: Balanced rearrangements not detected.
	Sanger sequencing	x	x	○	x	x	x	Pros: Useful for familial SRS cases (e.g. <i>IGF2</i> , <i>CDKN1C</i> ). Cons: Not suitable for a large gene.
	Exome Sequencing	Δ	Δ	○	○	x	x	Pros: Cost and time effective. Cons: Misses non-exonic regions.
	Whole genome sequencing	○	○	○	○	x	x	Pros: Investigates genome-wide in single-base resolution. Cons: Currently not cost effective for routine diagnostic use.
DNA Methylation	Bisulfite sequencing	Δ	x	x	x	○	Δ	Pros: Quantitative and easy. Cons: Takes a long time, cloning required. Not suitable for testing many loci.
	MS PCR	Δ	x	x	x	○	Δ	Pros: Quick and easy. Cons: Semi-quantitative. Does not distinguish UPD and CNV.
	MS Pyrosequencing	Δ	x	x	x	○	Δ	Pros: Quantitative. Cons: Requires pyrosequencing platform. Not suitable for testing many loci.
	MS qPCR	Δ	x	x	x	○	Δ	Pros: Quantitative. Cons: Not single-base resolution.
	MS S <sub>Nu</sub> PE	Δ	x	x	x	○	○	Pros: Cost and time effective. Cons: Only tests selected regions.
	High density methylation array	Δ	x	x	○	○	○	Pros: Cost and time effective method to test multiple loci. Could be used to check UPD if iDMRs are involved. Cons: Bioinformatics analysis still not hands-on for routine diagnostics.
	Enrichment NGS (e.g. MeDIP-seq)	Δ	x	x	○	○	○	Pros: Investigates genome-wide. Distinguish 5mC and 5hmC. Cons: Not single base resolution. Bioinformatics analysis still not hands-on for routine diagnostics.
	Whole genome bisulfite sequencing	Δ	○	Δ	○	○	○	Pros: Investigates genome-wide. Cons: Currently not cost effective for diagnostic use.
Genomic and DNA Methylation	MS-MLPA	x	Δ	x	x	○	Δ	Pros: Simultaneously detect ICR1 and ICR2 methylation and CNV at Chr11p15.5. Cons: Misses other DMRs.

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## Ref annotations

\*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. *Journal of medical genetics* 52(7), 446-453 (2015).

- This paper describes the latest clinical scoring system for SRS diagnosis, with some advantage over previously described systems.

\*Eggermann T, Netchine I, Temple IK *et al.* Congenital imprinting disorders: EUCID.net - a network to decipher their aetiology and to improve the diagnostic and clinical care. *Clinical epigenetics* 7(1), 23 (2015).

- This paper describes the EUCID.net (European network of congenital imprinting disorders) consortium, which is aimed to promote better clinical care and scientific investigation of imprinted disorders (IDs). This also have a good summary of imprinting disorders.