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Genome-wide methylation analysis in Silver-Russell syndrome patients

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Abstract

Silver-Russell Syndrome (SRS) is a clinically heterogeneous disorder characterised by severe *in utero* growth restriction and poor postnatal growth, body asymmetry, irregular craniofacial features and several additional minor malformations. The aetiology of SRS is complex and current evidence strongly implicates imprinted genes. Approximately half of all patients exhibit DNA hypomethylation at the *H19/IGF2* imprinted domain, and around 10% have maternal uniparental disomy of chromosome 7. We measured DNA methylation in 18 SRS patients at >485,000 CpG sites using DNA methylation microarrays. Using a novel bioinformatics methodology specifically designed to identify subsets of patients with a shared epimutation, we analysed methylation changes genome-wide as well as at known imprinted regions to identify SRS-associated epimutations. Our analysis identifies epimutations at the previously characterised domains of *H19/IGF2* and at imprinted regions on chromosome 7, providing proof of principle that our methodology can detect DNA methylation changes at imprinted loci. In addition we discovered two novel epimutations associated with SRS and located at imprinted loci previously linked to relevant mouse and human phenotypes. We identify *RBI* as an additional imprinted locus associated with SRS, with a region near the *RBI* DMR hypermethylated in 13/18 (~70 %) patients.

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Authors' contributions

A.R.P. generated the in-house Illumina[®] 450k human methylation microarray data, further developed and implemented the data analysis approach, performed most of the data analyses, wrote the manuscript and prepared Tables and Figures. M.I. performed replication analysis using bisulfite mutagenesis analyses of selected regions, collated SRS patient information, and prepared some of the Tables. S.B. prepared DNA sample libraries for analysis on the Illumina[®] 450k human microarray. J.F. collated SRS patient information, and with W.P., extracted DNA from some of the control blood samples, and prepared MeDIP libraries. S.A.-A. provided patient information and performed locus-specific test for DNA methylation at the *H19* ICR1 in patient samples. P.S. provided intellectual contributions. R.S. conceived the data analysis approach, created the original R implementation, consulted on its further development, performed some data analyses, and wrote the manuscript. G.M. contributed to the conception and design of the experiments and provided SRS patient sample DNAs. R.J.O. contributed to the conception and design of the experiments and wrote the paper. All authors contributed to the final version of the paper.

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Competing interests

The author(s) declare that they have no competing interests

We also report 6/18 (~33 %) patients were hypermethylated at a CpG island near the *ANKRD11* gene. We do not observe consistent cooccurrence of epimutations at multiple imprinted loci in single SRS individuals. SRS is clinically heterogeneous and the absence of multiple imprinted loci epimutations reflects the heterogeneity at the molecular level. Further stratification of SRS patients by molecular phenotypes might aid the identification of disease causes.

Keywords

Silver-Russell syndrome; global DNA methylation; genomic imprinting; CpG islands

Background

Genome-wide studies are powerful tools that have been used successfully in the identification of genetic alterations associated with human disease (Koboldt et al. 2013). Nevertheless, a major challenge remains in elucidating the role of the epigenome, which directs important cell-type specific functions during development and may therefore play a role in human disease pathology. DNA methylation can be measured genome-wide using a variety of approaches including promoter-focused studies and global methylation assays at base pair resolution across the human genome. Each approach provides advantages and disadvantages in the detection of DNA methylation differences (reviewed in (Meaburn and Schulz 2012; Bock 2012)). Here we interrogate DNA methylation at ~485 000 CpGs genome-wide in patients with Silver-Russell syndrome (SRS) [OMIM180860], a disorder with no single overarching aetiology but with evidence for a having genetic and/or epigenetic causative components. We identify regions of methylation difference in patients compared to control samples, in an effort to improve the understanding of this disorder at the molecular level, including a comprehensive re-examination of its association with genomic imprinting.

SRS is characterised by intrauterine and/or postnatal growth restriction ($-2SD$ from the mean), relative macrocephaly, typical facial appearances such as triangular-shaped face and frontal bossing, and body asymmetry (Price et al. 1999). Other abnormalities include fifth finger clinodactyly, café au lait spots, male genital anomalies and speech delay (reviewed in (Wakeling et al. 2010)). SRS belongs to a group of ‘imprinting disorders’, which have an estimated incidence of 1 in 7,000 (Abu-Amro et al. 2010). Genomic imprinting involves epigenetic mechanisms whereby the expression of a gene is restricted to either the paternal or the maternal allele, resulting in parent-of-origin-specific monoallelic expression. This is primarily controlled by differentially methylated regions (DMRs) comprised of CpG-rich sequence where the methylation state of the cytosine residues depend on the parent-of-origin of the allele. A trend has been observed whereby paternally expressed genes promote fetal growth, whereas maternally expressed genes restrict fetal growth (Moore and Haig 1991). Human imprinting disorders are caused by alterations of gene expression dosage as a result of chromosome abnormalities or methylation defects (epimutations), often exhibiting abnormal growth phenotypes (Ishida and Moore 2013).

Previous investigations of both the potential genetic and epigenetic aetiologies of SRS have revealed its heterogeneous nature (Abu-Amro et al. 2010). To date, two major molecular

defects have been described. The first is that maternal uniparental disomy of chromosome 7 (mUPD7) is detected in approximately 10% of patients (Gicquel et al. 2005; Preece et al. 1997). mUPD7 results in over-expression of maternally expressed imprinted genes and/or loss of paternally expressed imprinted genes from the entire chromosome 7. In contrast, paternal UPD7 patients do not exhibit distinct growth phenotypes (Hoglund et al. 1994; Pan et al. 1998). Both hetero- and isodisomic mUPD7 SRS cases have been identified, suggesting the SRS phenotype may be a feature of aberrant imprinting rather than the exposure of a recessive allele (Preece et al. 1999).

Despite an extensive search of numerous individual candidate genes both on chromosome 7 and other human chromosomes, specific genes with a functional role in SRS have not been identified (Abu-Amro et al. 2010). The second epigenetic defect associated with SRS is hypomethylation of the *H19/IGF2* DMR, known as imprinting control region 1 (*H19* ICR1) at 11p15, detected in almost half of the assayed SRS patient cases (Gicquel et al. 2005). Chromosome region 11p15 harbours two adjacent imprinted domains, each controlled by its own ICR. ICR1 is found on the telomeric side, regulating the parent-of-origin specific monoallelic expression of the paternally expressed insulin-like growth factor 2 (*IGF2*) gene, a growth promoter, and of the maternally expressed non-coding RNA *H19*, a growth suppressor. ICR1 is normally methylated only on the paternal allele, and when hypomethylated, has been associated with reduced *IGF2* expression and biallelic expression of *H19* (Gicquel et al. 2005). Human and mouse studies provide strong evidence that this epimutation likely contributes to the growth restriction phenotype in SRS (Lee et al. 2010).

Recent studies have revealed that approximately 10% of SRS patients with ICR1 hypomethylation also have additional methylation defects (multi-locus methylation defects (MLMD)) present at other imprinted loci. These are found at both maternally and paternally methylated DMRs, strongly suggestive of aberrant imprint establishment or maintenance (Azzi et al. 2009; Eggermann et al. ; Turner et al. 2010; Court et al. 2013; Eggermann et al. 2014). However, these studies only focussed on selected regions. For genome-wide studies, Penaherrera et al. investigated 1,505 CpG sites of 22 SRS patients using the Illumina® Golden Gate methylation array, but detected no additional common methylation defects other than ICR1 hypomethylation (Penaherrera et al. 2010). Kannenberg et al. studied 27,500 CpG sites using the Illumina® HumanMethylation27 array and compared 18 SRS patients and 9 small-for-gestational-age children. This study revealed that up to 73% of patients with ICR1 hypomethylation have MLMD at other imprinted loci. However, neither study found recurrent methylation defects outside of ICR1 (Kannenberg et al. 2012).

The molecular features of the remaining ~40% of SRS patients still remain to be defined. Importantly, the hypomethylation at the *H19* ICR1 associated with half of the SRS patients is detectable in blood. Epigenetic factors such as DNA methylation are generally tissue-specific and accordingly, detection of an epigenetic defect in human disease is difficult due to inaccessibility to the relevant affected tissues. The presence of this hypomethylated allele in blood provides a rationale to search for additional novel methylation differences between SRS patients and controls in this tissue.

Materials and methods

SRS DNA samples

Eighteen SRS patients were included in the DNA methylation microarray study, as previously described by (Preece et al. 1997). Peripheral blood DNAs from SRS patient DNA samples were prepared using a phenol-chloroform solution (Sigma®). SRS patients fulfilled at least three of the five key criteria (birth weight $-2SD$ from the mean, postnatal growth $-2SD$ from the mean, relative macrocephaly, body asymmetry, typical facial features) (Price et al. 1999). The cohort consisted of nine males and nine females, ages 0.84-20.32 years at the time of assessment. Several patients were previously identified to have ICR1 hypomethylation (n=2) using a methylation-sensitive RFLP PCR assay (bis conF: 5'-gtagggttttggtaggtatagag -3', bis conR: 5'-cttaataaccraaacrtttccac -3'), the restriction enzyme used was TaqI (TCGA) (Cooper et al. 2005). Three SRS patients in the cohort were diagnosed with mUPD7, all remaining patients had a normal karyotype (detailed patient information is summarized in Table S1).

Control DNA samples

Six white European individuals were included in the study as in-house control samples, DNA was prepared from blood using a Ficoll®-density gradient. Informed consent was obtained from all participants and the study was approved by the Joint Research Ethics Committee of Great Ormond Street Hospital and the Institute of Child Health (approval 1278). 35 additional control methylation profiles from three additional studies were taken from the Gene Expression Omnibus (GEO) studies GSE42865, GSE32148 and GSE35069. Ages of control samples from GSE42865 were obtained by personal communication. The age of all controls from GSE35069 were assigned to be 38 (midpoint of 25-51), and the age of sample GSM796696 was assigned as 54 (average of study GSE32148) (Table S2). SRS patients without age data were assigned as 6 (average of all other SRS samples). This was done for the purpose of linear modelling.

Genome-wide DNA methylation detection

Infinium HumanMethylation450 BeadChip

This array interrogates ~485,000 methylation sites per sample at single-nucleotide resolution. It covers 99% of RefSeq genes, with an average of 17 CpG sites per gene region distributed across the promoter, 5'UTR, first exon, gene body, and 3'UTR. It covers 96% of CpG islands, with additional coverage in island shores and the regions flanking them. 18 SRS patient samples plus six control samples were analysed by this method on two BeadChip arrays run concurrently. 300ng of DNA was quantified using a QUBIT™ fluorometer and used to generate Infinium libraries according to the manufacturers instructions http://www.illumina.com/products/methylation_450_beadchip_kits.ilmn, sodium bisulfite conversion was performed using the EZ DNA methylation kit (Zymo Research). Arrays were scanned on an Illumina® BeadArray Reader. All array data were deposited in GEO under the accession number GSE55491.

MeDIP-seq

MeDIP-seq libraries were prepared using DNA extracted using the same methods as described previously. MeDIP DNA libraries were prepared for multiplexed-paired-end sequencing on the Illumina GAIIx platform as follows. DNA samples were quantified by Qubit DS DNA analysis and 6µg of DNA was sonicated to obtain an average fragment size of ~200 bp using the diagenode bioruptor (Diagenode, Belgium). Each sample was sonicated for 6 cycles of 10 mins (30s on/off) cycle at +4°C. Next generation sequencing libraries were prepared from sonicated DNA samples using the NEBNext DNA library preparation kit (NEB, USA). MeDIP was performed using 4µg of each library, using a previously described method (Puszyk et al. 2013). All samples (including inputs) were then amplified by ligation mediated PCR and DNA libraries were indexed to allow to pooling of 12 libraries for multiplexed sequencing of samples. The SRS cohort consisted of eight patients including two patients analysed using the beadchip array (patient details summarized in Table S1). The control group (n=5) consisted of 3 males and 2 females with mean age of 25.8 +/- 9.52 years. Processed MeDIP data in the form of BED9 and BigBed files can be downloaded from the following web address: <https://atlas.genetics.kcl.ac.uk/~rschulz/MeDIPseqSRS>

Locus-specific Methylation analysis using bisulfite PCR and sequencing

Bisulfite sequencing of the *RBI* locus was carried out on 12 SRS patients and six control whole blood DNA samples. Six of these SRS patients who showed hypermethylation at cg13389575 probe CpG, were included as technical verification, and six new SRS samples were included to screen for additional patients with hypermethylation. 1µg of DNA samples were treated with sodium bisulfite and purified using EZ DNA Methylation-Gold™ Kit (Zymo Research). Bisulfite PCR primers were designed using MethPrimer program (<http://www.urogene.org/methprimer/> [last accessed 10.06.14]), the primer sequences in 5' to 3' direction are as follows: *RBI_F*: gggatttatatgtaataggagttttta and *RBI_R*: taaaaaaaacaaaactaccctccc. Cloning of the PCR products was carried out with TOPO® TA Cloning kit (Invitrogen) following manufactures' instructions. An average of 27 colonies were picked per sample and sequenced using BigDye® Terminator v1.1 Cycle Sequencing kit (Applied Biosystems). Sequencer 4.10.1(Gene Codes) and BiQ Analyzer programmes (<http://biq-analyzer.bioinf.mpi-inf.mpg.de/> [last accessed 10.06.14]) were used for visualisation and quality control of DNA methylation data. Clones with C-T conversion of less than 98% were removed from the analysis for accurate quantitation of methylation levels.

Bioinformatics analysis

Experimental design and Illumina® HumanMethylation450 Beadchip data processing

Illumina® background-subtracted intensity data from the Illumina® HumanMethylation450 Beadchip were exported from Beadstudio, all subsequent statistical analyses were performed in R (version 2.15.2) unless explicitly stated. No normalisation was applied to correct for previously identified differences between the Type I and Type II assay types. Instead, the different assay types as well as their combinations with the two colour channels were treated as independent experiments. Specifically, intensity values from the array were split into six

separate datasets: Type I red methylated, Type I red unmethylated, Type I green methylated, Type I green unmethylated, Type II green (methylated), and Type II red (unmethylated). For a full description of the Infinium 450k array chemistry see Bibikova et al (Bibikova et al. 2011). Each dataset was independently subjected to between-array quantile normalisation. In addition to microarray data generated in-house, we used intensity values taken from 35 additional genome-wide methylation profiles deposited in GEO (Table S2). 474 highly polymorphic probes not present in all the GEO curated datasets were excluded from the analysis. Methylation analyses were performed using the limma package (Smyth 2005). To assess changes in methylation at each probe we used a linear model of the intensity levels in terms of the following experimental factors: disease state, sex, study, blood cell preparation (whole blood or PBMCs) and age. We ascertained that blood cell preparation had to be defined as an experimental factor since when we performed the linear modelling without this factor, we saw inflation of the test statistics (Figure S1). Defining blood cell preparation as an experimental factor allows us to control for cell-specific differences in methylation. Previous studies have detected blood cell-specific variation in methylation (Reinius et al. 2012).

Each SRS patient was tested individually versus 41 ‘control methylation profiles’ using a round-robin approach, performing independent linear modelling and testing for methylation changes on each of the six datasets. After modelling, all test P-values (across all six datasets) for the disease state coefficients were multiple testing-corrected using the Benjamini-Hochberg method (Benjamini and Hochberg 1995). Each CpG was thus assigned two fold changes and two corrected P-values, one for each of the methylated and unmethylated moieties (Figure 1, A).

For an individual CpG in a SRS patient to be designated as either hypomethylated or hypermethylated versus controls, it had to pass one of three logical tests: either (1) both methylated and unmethylated intensities exhibited a significant change ($FDR < 50\%$) and the intensity values moved in opposite directions, i.e., when the methylated intensity increased, the unmethylated intensity decreased, or (2) the methylated intensity exhibited a significant change ($FDR < 50\%$) and the unmethylated intensity exhibited no significant change ($FDR > 70\%$), or (3) the unmethylated intensity exhibited a significant change ($FDR < 50\%$) and the methylated intensity exhibited no significant change ($FDR > 70\%$) (Figure 1, B). Using these three logical indicators, each CpG was assigned a score of -1 (hypomethylated), $+1$ (hypermethylated), or 0 (no significant change in methylation) for each individual SRS patient. The sum of the CpG scores across the 18 patients is the group-wise CpG score, ranging from -18 to 18 (Figure 1, C). For example, a score of -18 would indicate that the CpG was significantly hypomethylated in all SRS patients. Adjacent CpGs were subsequently considered in combination depending on their genomic context. For example, CpGs within or near a specific CpG island (additional data file 1), gene promoter region (additional data file 2) or gene body region (additional data file 3) were considered together, and their score average became the ‘island/promoter/gene body score’ (Figure 1, D). Only distinct genomic regions containing two or more CpGs were considered in our analyses. Control methylation profiles were used to define the normal range of genomic region scores (Figure 2 A,B,C). Regions were considered differently methylated in SRS if the region score was outside of the 0.1th-99.9th percentile range observed in controls.

Chromosome plots of CpG islands showing changes in methylation in SRS patients were prepared using the ideographica package (Kin and Ono 2007).

RnBeads bioinformatics analysis

Raw intensity values from the in-house microarray, and from GEO were subjected to between-array quantile-normalisation, as above, separately for each assay type and colour channel, and beta-values were computed from the normalised intensities. Within-array BMIQ normalisation was used to correct for technical differences between Type I and Type II probes. Finally, a groupwise comparison was performed between the SRS and control cohorts. To assess methylation differences at each probe we used a linear model of beta-values in terms of the following experimental factors: disease state, sex, study, blood cell preparation (whole blood or PBMCs) and age.

MeDIP bioinformatics analysis

Bioinformatic analysis was performed using the same statistical method as in Proudoun *et al.* (Proudoun et al. 2012). However, rather than a group-wise comparison, a round robin analysis was performed comparing each SRS patient individually versus the control group.

SRS methylation differences at imprinted DMRs

Overrepresentation of differences in methylation in SRS at or within 100 kb of a known imprinted DMRs was tested using the chi-squared test with Yates' correction, and was calculated both including and excluding all CpG islands on chromosome 7 and within 100 kb of the *H19* ICR1, due to the presence of mUPD7 and *H19* ICR1 hypomethylated SRS patients. A list of known imprinted DMRs was obtained from WAMIDEX (downloaded 2010) (Schulz et al. 2008).

To test for overlap between CpG islands that show differences in methylation in SRS and known imprinted regions of the human genome, coordinates of imprinting DMRs were taken from WAMIDEX and merged with coordinates of imprinted genes from geneimprint (geneimprint.org, downloaded 2010). These two sets of regions were considered in a hierarchical fashion: entries from geneimprint were only considered if no imprinted DMR had been identified for a particular imprinted locus (Table S3). Using the intersect tools in Galaxy (Giardine et al. 2005), we scanned for CpG islands that displayed methylation changes in SRS and that occurred within 100 kb of an imprinted DMR or imprinted gene region.

Methylation changes at gene promoters and in gene bodies

Gene promoter and gene body methylation was assayed independently and the results combined based on a common Entrez ID to identify genes that displayed differences in methylation in SRS at both the promoter and in the gene body. The proportional Venn diagram to illustrate overlap was generated using the Vennrable package implemented in R.

Ontological analysis

Genes that displayed differences in methylation in SRS at the promoter or within the gene body were assayed for overrepresentation in specific biological processes (BP) or molecular functions (MF) using the GOSTats package implemented in R (Falcon and Gentleman 2007).

RESULTS

A novel method for analysing 450k Infinium methylation microarray data

The 450k Infinium microarray platform employs two different assay types (Type I and Type II) and two different colour channels (red and green) to measure DNA methylation. Type I relies on two distinct probes, measuring the unmethylated (U) and methylated (M) moieties of a CpG in a sample, respectively. A proportion of Type I assays uses the red colour channel, while the remainder use the green channel. Type II assays rely on a single probe returning a measurement of the U and M moieties of a CpG in the red and green channels, respectively. The distributions of the measured fluorescence signal (intensity) systematically differ between the two moieties but also between assay types and colour channels, i.e., for both biological and technical reasons (Teschendorff et al. 2013; Touleimat and Tost 2012; Staaf et al. 2008; Maksimovic et al. 2012).

Instead of manipulating the intensity values to try to specifically neutralise the technical biases, we treated each array as a composite of six distinct array types (Type I red U, Type I red M, Type I green U, Type I green M, Type II U, Type II M) and performed quantile-normalisation across samples, linear modelling and testing for significant intensity differences between samples separately for each array type (using limma (Smyth 2005)). All resulting test P-values were globally multiple testing corrected using the Benjamini-Hochberg method of controlling the false discovery rate (FDR) (Benjamini and Hochberg 1995).

Consequently, for each assayed CpG, two test results, one for each of the U and M moieties, had to be combined to generate a value and corresponding score (Figure 1). We considered a CpG to be significantly hypermethylated (scored as +1) if M significantly (FDR < 50%) increased and U significantly decreased, or if M significantly increased and U showed no significant change (FDR > 70%), or if U significantly decreased and M showed no significant change; analogously for a significantly hypomethylated CpG (scored as -1). Otherwise the CpG was considered as not differently methylated (scored as 0) (Figure 1,B).

Instead of a group-wise comparison of all SRS patients (n= 18) versus all non-SRS control samples (n= 41), we chose a round-robin approach, comparing each individual SRS patient with the group of non-SRS controls so that test results for individual patients were available for detailed inspection of group-wise significant results (Figure 3). For each CpG, the test scores were added across patients (Figure 1, C). The CpG scores were averaged across the CpGs within defined genomic regions (e.g., CpG islands) containing at least two scored CpGs to generate per-region scores (Figure 1, D). The same round-robin approach was applied to compare each non-SRS control sample with the remaining controls to generate baseline distributions for CpG and genomic region scores. We then considered a CpG or

genomic region to have a significant score in the SRS group if the score fell outside of the range defined by the 0.1th and 99.9th percentiles of the baseline distribution.

SRS-associated DNA methylation changes are distributed across the genome

The numbers of significantly hypermethylated and hypomethylated CpGs in each SRS patient were closely correlated with one another (Pearson's correlation = 0.99, $P < 0.001$) and not significantly different from each other (Student's t test, $P = 0.74$), in other words methylation changes in SRS patients relative to controls were comprised approximately equally of losses and gains of methylation (Figure S2). One exceptional patient DNA sample (SRS 6) appeared to be an outlier with an excess of methylation changes by comparison to the 17 other patient samples analysed in this study, but hypo- and hypermethylated CpGs were again equally frequent. On average, 6667 (1.4%) of CpGs showed significant changes in methylation in the SRS patient group compared to the control group.

We summarised methylation changes across CpG Islands, gene promoters and gene bodies where the role of DNA methylation in relationship to gene expression has been characterised in most detail (Jones 2012), by averaging the scores obtained for all CpGs in the respective genomic region. Again, there was no obvious skew towards global hypo- or hypermethylation at the level of CpG islands, gene promoters or gene bodies (Figure 2 A,B,C). The genomes of these SRS patients therefore are not globally hypo- or hypermethylated, consistent with previous findings (Dias et al. 2012).

We found 313 CpG islands that showed significant differences in methylation in the SRS patient group (Table S4, Figure 2, A), an almost 40-fold increase over the control group. These CpG islands were distributed across all chromosomes, including the sex chromosomes (Figure 4). Chromosome 19 had the highest density of methylation changes in CpG islands, most likely due to its particularly high gene density. Interestingly, CpG islands with methylation differences appeared to cluster together into regions with runs of multiple either hypo- or hypermethylated CpG islands, rather than alternating between hyper- and hypomethylated islands. This is especially obvious for but not limited to chromosome 19 and may represent the coordinated regulation of multiple loci in larger genomic regions.

Specific imprinted regions are affected by methylation changes in SRS

SRS is one of several disorders associated with imprinted regions (Ishida and Moore 2013). Approximately 50% of SRS cases display hypomethylation at the *H19* ICR1, while a further 5-10% of SRS patients exhibit mUPD7 (Gicquel et al. 2005; Preece et al. 1997). We determined the prevalence of SRS-specific differences in methylation at imprinted loci in a genome-wide, systematic way. In order to reduce the risk of systematic errors introduced by genetic variation within probes, we visually inspected probes that displayed differences in methylation for overlap with common variants (>5% minor allele frequency).

We hypothesised that methylation changes in CpG islands may occur more frequently at or near (within 100 kb of) known imprinted DMRs than expected by chance. We found that this was the case (Chi-squared test with Yates' correction: $P < 0.0001$; Table S5). However, this result is subject to an ascertainment bias because of the known mUPD7 and *H19* ICR1

molecular phenotypes in our SRS cohort. When we excluded CpG islands and DMRs on chromosome 7 and within 100 kb of the *H19* ICR1, the result was no longer strictly significant ($P = 0.0545$; Table S5) with only two (*RB1* and *MCTS2*) of the 20 known DMRs, showing differences in methylation in our SRS patient group. In addition, the distance between the the *MCTS2* DMR and the CpG island with methylation changes was further than 25 kb. If we are being conservative, and assess only regions within 25 kb of a known imprinted DMR, the result is not significant ($P = 0.2481$). When we extended our analysis to all reportedly imprinted genes, even those for which no imprinted DMR has yet been defined (for the full list of regions see Table S3), we only found one additional methylation difference at a CpG island within 100 kb, near the *ANKRD11* gene. Thus, we found no evidence of an overarching methylation phenotype affecting all or a large proportion of imprinted DMRs in SRS. However, the methylation change at *RB1* may be additional molecular phenotypes by which to stratify SRS patients (Table 1).

Hypomethylation at the *H19* ICR1 may be more common in SRS than previously thought

Hypomethylation at the *H19* ICR1 region was detected in six SRS patients at multiple CpGs across the region, while 12 samples exhibited no obvious methylation changes (Table 1, S6). This was at odds with previous tests for *H19* ICR1 hypomethylation, which failed to detect hypomethylation in three of these patients (with one untested). Our results therefore suggest that the methylation-sensitive RFLP PCR method, which assays a single CpG site (see methods), failed to correctly detect *H19* ICR1 hypomethylation in a significant proportion of cases. Because the entire *H19* ICR displays methylation differences in SRS this result highlights the limitation of extrapolating the methylation status of the *H19* ICR1 from the measurement of DNA methylation at a single CpG dinucleotide.

Detection of hypermethylation of the imprinted DMRs on chromosome 7 provides further proof-of-principle for this analysis approach

The CpG islands corresponding to the imprinted DMRs at *GRB10*, *PEG10* and *MEST* were identified as significantly hypermethylated in the SRS patient group. This result was due to three mUPD7 patients in the SRS group ($n = 18$). mUPD7 individuals carry two maternal and no paternal copies of chromosome 7 so that the maternally methylated imprinted DMRs on chromosome 7, as compared to the control group, were expected to appear hypermethylated in individual mUPD7 patients. Our analysis approach was sufficiently sensitive to detect these methylation differences present in only 1/6th of the patients. Non-mUPD7 patients did not harbour significant methylation differences at these imprinted DMRs (Table 1, S7). These results, combined with the results for the *H19* ICR1, provide proof-of-principle that this analysis approach is capable of identifying methylation changes with high sensitivity.

Hypermethylation at the *RB1* imprinted DMR in >70% of SRS cases

We detected significant hypermethylation of a CpG island 1087 bp from the *RB1* imprinted DMR on chromosome 13 in the SRS patient group (Table 1). On an individual basis, the CpG island was significantly hypermethylated in 11 of 18 SRS samples (55%). Closer inspection of the methylation scores for the CpGs in the island showed that two adjacent CpGs in the island shore (cg13389575 at chr13:48890413 and cg11882053 at

chr13:48890459) contributed predominately to the hypermethylation score of the island. At least one of these two CpGs was significantly hypermethylated in 13 SRS patients (Table S8, Figure S3), with an average increase in methylation of approximately 10%. The 13 patients did not present with a clinical phenotype that distinguished them from the five remaining patients (Table 1). Hypermethylation at *RB1* occurred in patients with other molecular anomalies (e.g., mUPD7, *H19* ICR1 hypomethylation). Interestingly, six patients also exhibited hypomethylation of CpG cg24342013, nearer to the imprinted DMR, suggesting a potentially complex pattern of methylation changes at this locus in SRS. We propose DNA hypermethylation in the chr13:48890413-48890459 region to be of interest as a molecular phenotype associated with SRS. These probes are located in the CpG island shore, peripheral regions of the island that have been associated with environmental specific dynamic changes in methylation (Yang et al. 2013).

Hypermethylation at the *ANKRD11* locus in SRS

We identified a CpG island located 25 kb downstream of the *ANKRD11* gene that was significantly hypermethylated in the SRS patient group (Table 1, S9, Figure S4). The CpG island coincides with a piRNA gene cluster (piR-50317; GenBank: DQ583205.1), a class of small RNAs involved in chromatin remodelling (Peng and Lin 2013). Individually, six of the 18 SRS patients were significantly hypermethylated at two or more CpG probes located within the island. Three CpGs, located within the island, contributed predominately to the hypermethylation score of the island and when considered together displayed an average increase in methylation of 1.2% in the six SRS patients. It is questionable whether this small increase in methylation is biologically meaningful. However, because the *ANKRD11* locus is implicated in both 16q24.3 microdeletion syndrome, with haploinsufficiency of *ANKRD11* (Willemsen et al. 2010), and in KBG syndrome where several mutations in *ANKRD11* are observed (Sirmaci et al. 2011), we considered this observation interesting. Both syndromes exhibit significant phenotypic overlap with each other and with SRS, including short stature, triangular face, and fifth-finger clinodactyly (Spengler et al. 2013). Although this locus is designated as an imprinted region in the geneimprint database (geneimprint.com), the imprinted status of *ANKRD11* has not been confirmed. *ANKRD11* is ~70 kb downstream of *CDH15* which is imprinted in a tissue-specific manner in mouse, but imprinting has not been observed in human (Proudhon et al. 2012).

Replication of significant hits using MeDIP-seq and Bisulfite analysis

In order to confirm the validity our findings, we performed a replication analysis in eight patients with SRS using MeDIP-seq applied to blood samples, which assays DNA methylation genome-wide. Our replication cohort consisted of two SRS individuals analysed using the Illumina® 450k methylation microarray and 6 additional SRS patient samples, each compared separately to a set of five normal controls. Analysis of methylation at the *H19* ICR1 using MeDIP-seq revealed hypomethylation in 3 of 8 patients (Figure S5), providing proof-of-principle for this method to detect known epimutations in our SRS cohort.

The MeDIP-seq analysis additionally revealed that 4/8 (50%) individuals were hypermethylated at the *RB1* DMR (FDR < 50%), supporting our microarray-based findings

(Figure S6). Of the two samples analysed using both methods we detected hypermethylation at the *RB1* DMR in SRS 17 using both the microarray and MeDIP platforms. SRS 5 was not designated as hypermethylated using the microarray; however, hypermethylation was detected using MeDIP-seq.

Further confirmation of hypermethylation at the *RB1* DMR was performed in 12 SRS patients using bisulfite conversion followed by PCR, cloning and sequencing, with results again supporting those generated by the array analysis (Figure S7). Eight of 12 samples showed higher methylation levels at the location of probe cg13389575 when compared to the control methylation level (Table S10). Of the six samples analysed on both platforms (SRS12, SRS17, SRS22, SRS26, SRS30, SRS31), four recapitulated the hypermethylation phenotype observed at this position using the 450k microarray (not SRS31 and SRS26). The degree of methylation difference at this location was modest and there was no difference in methylation levels when the average of ten CpG dinucleotides across the region was compared instead, likely indicating a CpG-specific methylation effect, or technical sensitivity differences between the assays.

Methylation changes within genes in SRS

In a separate analysis, we identified 174 gene promoters and 277 gene bodies that displayed significant differences in methylation in the SRS patient group (Figure 2,D). Fifteen genes showed differences in methylation at both the promoter and in the gene body (Table 2). We inspected the methylation changes at the single CpG level for each of the 15 genes, and excluded genes where CpGs in the first exon heavily influenced the score of both the gene promoter and the gene body. The remaining two genes were *NRBP1* and *MSS51*. For *NRBP1*, we observed hypomethylation at the CpG island gene promoter and hypermethylation in the gene body. This pattern suggests that *NRBP1* may be over-expressed in some SRS patients. However, this methylation pattern at *NRBP1* was only consistently observed in two of the SRS patients. *MSS51* was hypomethylated at both the (non-CpG island) promoter and in the gene body in four SRS patients. *MSS51* therefore may also be over-expressed in some SRS patients, but the relationship between DNA methylation and gene expression levels is less well characterised and overall more variable for genes with non-CpG promoters (Jones 2012).

Comparison with group-wise analysis

To evaluate our novel bioinformatics pipeline, a comparison with a standard approach used to analyse Illumina® 450k microarray data was performed. We made a group-wise comparison of SRS versus control methylation profiles using between-array quantile-normalisation of signal intensities as before, within-array BMIQ normalisation of beta values, and linear modelling with limma including covariate factors as before within the RnBeads package (Assenov Y. 2014). This analysis detected no genome-wide statistically significant changes at the CpG, CpG islands or gene promoter/body level. This highlights the difficulty of detecting significant group-wise changes in methylation when the effect is only observed in a subset of individuals. Inspection of specifically the *RB1* DMR results obtained with RnBeads showed relative and nominally significant hypermethylation of the SRS group versus controls ($p=0.04$), consistent with the results of our original round-robin

analysis, while not achieving genome-wide statistical significance. With RnBeads, we obtained analogous results for the *H19* ICR1 and at the imprinted DMRs on chromosome 7 where a subset of our SRS patient cohort harbour known methylation changes that were not detected at a genome-wide significant level using the group-wise approach. Specific inspection of these regions again showed that the expected methylation changes were present in the results (Figure S8).

DISCUSSION

Global hypo- or hypermethylation is not a hallmark of SRS

The underlying aetiology of SRS is heterogeneous and has not been easy to elucidate. The syndrome re-occurs only very rarely in families, and it has been proposed to have complex genetic and/or epigenetic components based on an embryonic growth phenotype associated with several imprinted loci (Abu-Amro et al. 2010). Additionally, a case of monozygotic twins discordant for SRS has previously been reported, providing strong evidence that some instances of SRS can be explained by epigenetic differences alone (Gicquel et al. 2005). One possible hypothesis is that SRS patients display global disruption of DNA methylation with a genome-wide decrease or increase in DNA methylation. This study measured DNA methylation changes in 18 patients with SRS, compared to controls, at >450,000 CpGs. We did not detect a bias towards either hypomethylation or hypermethylation of DNA genome-wide, neither in terms of methylation changes at individual CpG probes nor at the level of CpG islands, gene promoters or gene bodies. We did detect many methylation differences that fall outside of the range observed in the control group, indicating that DNA methylation patterns in the blood of patients with SRS are overall more variable than those of non-SRS controls.

Methylation changes vary between individuals with SRS

We employed a novel round-robin approach to the analysis of DNA methylation microarray data generated on the Illumina® 450k Infinium platform. This approach was sensitive enough to detect significant methylation changes in our cohort of SRS patients even if the changes were limited to a relatively small subset of patients, and it allowed us to contextualise the detected methylation changes on a per-patient basis. The increased sensitivity of our round-robin analysis method was confirmed by comparing this approach to a conventional group-wise comparison that did not detect any genome-wide significant hits including known SRS-specific changes in methylation that are present in the cohort (*H19* ICR1 and Chromosome 7 DMRs). SRS is a clinically diagnosed syndrome with subgroups that share specific molecular anomalies (mUPD7, *H19* ICR1 hypomethylation), but for which no single overarching methylation defect is present in every patient. Our bioinformatic approach was specifically designed to detect methylation changes in subgroups of patients in a syndrome where molecular heterogeneity is expected. This method also circumvented the issues related to the combination of two different assay types and colour channels onto a single array platform. In addition, we substantiated the importance of controlling for differences in DNA extraction methods from blood when modelling methylation data, as illustrated by the inflation of the test statistic when the extraction method was not controlled for (Figure S1). This result was expected since

differences in methylation patterns have previously been identified between DNA extracted from peripheral blood mononuclear cells (PBMCs) and DNA extracted from whole blood (Reinius et al. 2012).

We did not detect a ubiquitous SRS epimutation. There are several possible explanations for this observation. Firstly, SRS phenotypes are a variable spectrum and a clinical diagnosis of SRS is based on a combination of several major and minor diagnostic criteria (Price et al. 1999; Wakeling et al. 2010). SRS therefore is likely phenotypically heterogeneous, representing a set of symptoms caused by several underlying molecular defects, including several distinct epigenetic defects. Our findings support this notion. In addition, the analysis of DNA methylation in blood likely yields results that are only partially representative of methylation differences that function to regulate growth and development of the fetus *in utero*. Using DNA extracted from blood makes the assumption that methylation changes from early developmental time points are preserved in the DNA of cells that have differentiated along the hematopoietic lineage. The rationale for this assumption is that hematopoietic stem cells are derived from the aorta-gonad-mesonephros region of embryonic mesoderm (reviewed in (Dzierzak and Speck 2008)). The embryonic mesoderm also gives rise to smooth and striated muscle, bone, cartilage, adipose tissue and the genitourinary system, all of which are involved in the SRS phenotypes (Price et al. 1999; Wakeling et al. 2010). However, the lack of common DNA methylation changes in all SRS cases may be because all common epigenetic differences occur early in development and are subsequently overridden by methylation changes that regulate cell differentiation, or are simply lost passively due to a lack of maintenance during cell division. For this reason we were particularly interested in imprinted DMRs because these are largely stable regions of DNA methylation during development and postnatal life (Woodfine et al. 2011). In particular, they tend not to be influenced by tissue-specific epigenetic reprogramming, as is demonstrated by the relative ease with which methylation changes at the *H19* ICR1 are detectable in DNA extracted from the blood of SRS patients (Gicquel et al. 2005).

Ontological analysis suggests most methylation differences detected in SRS patients are likely to be non-causative

CpG islands represent the most interesting genomic regions for examining differences in methylation in SRS because methylation changes in these regions directly influence the local epigenetic environment to regulate gene expression (reviewed in (Deaton and Bird 2011)). We found 313 CpG islands (1.2 % of CpG islands assayed) to be significantly differently methylated in SRS. Genes associated with these CpG islands were not over-represented for growth, skeletal development or muscle development ontologies as one might expect based on the SRS phenotype. However, we did observe over-represented ontologies associated with metabolism and transcription (Table S11). We propose that many of these methylation changes may therefore be a consequence of the symptoms of SRS, rather than being the underlying molecular cause. For example, one clinical observation in patients with SRS is a failure to feed properly during infancy. DNA methylation analysis in several tissue types in mouse demonstrate that calorie restriction can induce methylation changes (Chouliaras et al. 2012; Chen et al. 2013), and correlation between dietary intake and methylation has been shown for human leukocyte DNA (Gomes et al. 2012).

Imprinted regions and SRS

Methylation changes at imprinted regions have previously been described as epigenetic markers of SRS (Girardot et al. 2013). This study demonstrates multiple instances of methylation differences at imprinted regions in SRS patients, and 11 of 18 patients exhibit methylation changes at multiple imprinted domains, supporting the Multi-Locus Methylation Defect model previously proposed (Azzi et al. 2009; Eggermann et al. ; Turner et al. 2010; Court et al. 2013; Eggermann et al. 2014). However it should be noted that we also identified many instances of methylation changes in SRS patients compared to controls outside of imprinted regions. The best characterised epigenetic marker associated with SRS is at the *H19* ICR1. Our analysis detected this region as being hypomethylated in our patient cohort. When ranked by CpG island score, this region was 25th highest in terms of differences in methylation in SRS. Two SRS patients included in our study had previously been characterised as hypomethylated at *H19* ICR1 using a locus specific test. However, we detected hypomethylation in an additional four patients when we examined differences in methylation at the *H19* ICR1 in detail. DNA methylation changes had not been detected in three of these patients using this PCR test, and one patient had not been tested. This illustrates the limitations of assessing regional DNA methylation by measuring methylation at a single CpG. This may explain the large range in frequency of *H19* ICR1 hypomethylation reported in the literature (Blick et al. 2006; Gicquel et al. 2005; Eggermann et al. 2006), and has implications not just for SRS, but for other disorders in which hypomethylation is measured. The MS-MLPA (Holland) clinical test for hypomethylation measures methylation at four locations across the island, reflecting the need to assay multiple CpGs in order to ensure hypomethylation is accurately assessed (Figure S9). In addition we detected hypermethylation at known imprinted DMRs on chromosome 7 in patients with mUPD7, showing partial overlap with a chromosome-wide study of methylation in uniparental disomy 7 (Hannula-Jouppi et al. 2013).

The region adjacent to the *RB1* DMR may serve as an epigenetic mark for a subset of cases of SRS. We detected hypermethylation in the shore of a CpG island next to the *RB1* DMR in over half of the SRS patients analysed, irrespective of other reported molecular changes (mUPD7, *H19* ICR1 hypomethylation). Inspection of probes annotated at or near the differently methylated CpG island revealed that the signal was predominately due to two neighbouring CpGs, at least one of which was hypermethylated in 13/18 SRS patients analysed in this study, this observation was confirmed using both MeDIP-seq and Bisulfite analysis in replication studies.

Hypomethylation has previously been reported at the *RB1* DMR in an isolated case (Court et al. 2013). *RB1* is an imprinted gene preferentially expressed from the maternally derived allele (Kanber et al. 2009; Buiting et al. 2010) and is implicated in cell cycle progression, acting as a tumour suppressor (reviewed in (Weinberg 1995)). Preferential maternal expression of *RB1* may point towards a role for this gene in the regulation of growth and development that could be influenced by local epigenetic changes. *RB1* is implicated in several human cancers including retinoblastoma (Yandell et al. 1989), osteosarcoma (Sandberg and Bridge 2003), bladder cancer (Horowitz et al. 1990), and small cell lung carcinoma (Harbour et al. 1988). No increase in the frequency of *RB1*-related cancers have

been reported for SRS. The *Rb1* DMR is not present in the mouse (Rademacher et al. 2014), however, mice with a conditional deletion of *Rb1* in osteoblasts exhibit skeletal abnormalities that recapitulate some human SRS phenotypes (Gutierrez et al. 2008). *Rb1* together with imprinted genes like *Igf2* and *Grb10* are also implicated in the parent-of-origin-specific developmental defects observed in *Peromyscus* inter-species hybrids (Duselis and Vrana 2007).

Finally, we report a CpG island near *ANKRD11*, a gene predicted but not confirmed to be subject to genomic imprinting (geneimprint.com), which is hypermethylated in six SRS patients. The hypermethylated CpG island contains a piRNA cluster. piRNAs have been shown to regulate DNA methylation on the paternally inherited allele at the imprinted *Rasgrf1* locus in the mouse (Watanabe et al. 2011). This may be indicative of an altered epigenetic environment in the *ANKRD11* region in a subgroup of SRS patients, which may affect expression of *ANKRD11* and/or neighbouring genes contributing to the portions of the SRS phenotype that overlap with abnormalities seen in KBG and 16q24.3 microdeletion syndrome. It should be noted that this region is not identified as hypermethylated at genome-wide significance in the replication analysis using MeDIP-seq. We therefore propose it as an interesting region for further study.

Conclusions

We show that the DNA methylation profile of patients with SRS varies greatly from the range observed in controls, suggesting that methylation changes are playing a role in the pathology of SRS. Despite many methylation differences being observed, we do not observe global hypo- or hypermethylation nor do we observe a single common epimutation present in all patients in our SRS cohort. Instead we observe many regions where smaller subgroups of patients share methylation changes. Causative epimutations may result from aberrant function of trans-acting epigenetic modifiers during early development and drive the pathology of SRS. This mechanism of SRS pathology is evidenced by the presence of methylation changes at some imprinted regions across the genome that usually remain stable during development. In addition, the apparent co-regulation of large genomic regions observed in SRS are suggestive of irregular DNA methylation establishment and maintenance across the genome. The epimutations detected, could provide a tool to stratify SRS patients at a molecular level for further genetic and epigenetic classification.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

SRS	Silver-Russell Syndrome
ICR1	Imprinting Control Region 1
MLMD	Multi-Locus Methylation Defect
DMR	Differentially Methylated Region
FDR	False Discovery Rate
RFLP	Restriction Fragment Length Polymorphism
piRNA	Piwi-interacting RNA

References

- Abu-Amro S, Wakeling EL, Preece M, Whittaker J, Stanier P, Moore GE. Epigenetic signatures of Silver-Russell syndrome. *J Med Genet.* 2010; 47(3):150–154. doi:47/3/150 [pii] 10.1136/jmg.2009.071316. [PubMed: 20305090]
- Assenov Y., MF.; Lutsik, P.; Walter, J.; Lengauer, T.; Bock, C. Comprehensive Analysis of DNA Methylation Data with RnBeads. 2014. <http://rnbeads.mpi-inf.mpg.de>
- Azzi S, Rossignol S, Steunou V, Sas T, Thibaud N, Danton F, Le Jule M, Heinrichs C, Cabrol S, Gicquel C, Le Bouc Y, Netchine I. Multilocus methylation analysis in a large cohort of 11p15-related foetal growth disorders (Russell Silver and Beckwith Wiedemann syndromes) reveals simultaneous loss of methylation at paternal and maternal imprinted loci. *Hum Mol Genet.* 2009; 18(24):4724–4733. doi:ddp435 [pii] 10.1093/hmg/ddp435. [PubMed: 19755383]
- Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society.* 1995; 57(1):289–300. Series B.
- Bibikova M, Barnes B, Tsan C, Ho V, Klotzle B, Le JM, Delano D, Zhang L, Schroth GP, Gunderson KL, Fan JB, Shen R. High density DNA methylation array with single CpG site resolution. *Genomics.* 2011; 98(4):288–295. doi:10.1016/j.ygeno.2011.07.007. [PubMed: 21839163]
- Blik J, Terhal P, van den Bogaard MJ, Maas S, Hamel B, Salieb-Beugelaar G, Simon M, Letteboer T, van der Smagt J, Kroes H, Mannens M. Hypomethylation of the H19 gene causes not only Silver-Russell syndrome (SRS) but also isolated asymmetry or an SRS-like phenotype. *American journal of human genetics.* 2006; 78(4):604–614. doi:10.1086/502981. [PubMed: 16532391]
- Bock C. Analysing and interpreting DNA methylation data. *Nat Rev Genet.* 2012; 13(10):705–719. doi:nrg3273 [pii] 10.1038/nrg3273. [PubMed: 22986265]
- Buiting K, Kanber D, Horsthemke B, Lohmann D. Imprinting of RB1 (the new kid on the block). *Briefings in functional genomics.* 2010; 9(4):347–353. doi:10.1093/bfgp/elq014. [PubMed: 20551090]
- Chen PY, Ganguly A, Rubbi L, Orozco LD, Morselli M, Ashraf D, Jaroszewicz A, Feng S, Jacobsen SE, Nakano A, Devaskar SU, Pellegrini M. Intrauterine calorie restriction affects placental DNA methylation and gene expression. *Physiological genomics.* 2013; 45(14):565–576. doi:10.1152/physiolgenomics.00034.2013. [PubMed: 23695884]
- Chouliaras L, van den Hove DL, Kenis G, Keitel S, Hof PR, van Os J, Steinbusch HW, Schmitz C, Rutten BP. Prevention of age-related changes in hippocampal levels of 5-methylcytidine by caloric restriction. *Neurobiology of aging.* 2012; 33(8):1672–1681. doi:10.1016/j.neurobiolaging.2011.06.003. [PubMed: 21764481]
- Cooper WN, Luharia A, Evans GA, Raza H, Haire AC, Grundy R, Bowdin SC, Riccio A, Sebastio G, Blik J, Schofield PN, Reik W, Macdonald F, Maher ER. Molecular subtypes and phenotypic expression of Beckwith-Wiedemann syndrome. *European journal of human genetics : EJHG.* 2005; 13(9):1025–1032. doi:10.1038/sj.ejhg.5201463. [PubMed: 15999116]
- Court F, Martin-Trujillo A, Romanelli V, Garin I, Iglesias-Platas I, Salafsky I, Guitart M, Perez de Nanclares G, Lapunzina P, Monk D. Genome-wide allelic methylation analysis reveals disease-

- specific susceptibility to multiple methylation defects in imprinting syndromes. *Human mutation*. 2013; 34(4):595–602. doi:10.1002/humu.22276. [PubMed: 23335487]
- Deaton AM, Bird A. CpG islands and the regulation of transcription. *Genes & development*. 2011; 25(10):1010–1022. doi:10.1101/gad.2037511. [PubMed: 21576262]
- Dias RP, Bogdarina I, Cazier JB, Buchanan C, Donaldson MC, Johnston LB, Hokken-Koelega AC, Clark AJ. Multiple segmental uniparental disomy associated with abnormal DNA methylation of imprinted Loci in silver-russell syndrome. *The Journal of clinical endocrinology and metabolism*. 2012; 97(11):E2188–2193. doi:10.1210/jc.2012-1980. [PubMed: 22942387]
- Duselis AR, Vrana PB. Assessment and disease comparisons of hybrid developmental defects. *Hum Mol Genet*. 2007; 16(7):808–819. doi:10.1093/hmg/ddm025. [PubMed: 17339267]
- Dzierzak E, Speck NA. Of lineage and legacy: the development of mammalian hematopoietic stem cells. *Nature immunology*. 2008; 9(2):129–136. doi:10.1038/ni1560. [PubMed: 18204427]
- Eggermann T, Heilsberg AK, Bens S, Siebert R, Beygo J, Buiting K, Begemann M, Soellner L. Additional molecular findings in 11p15-associated imprinting disorders: an urgent need for multi-locus testing. *Journal of molecular medicine*. 2014 doi:10.1007/s00109-014-1141-6.
- Eggermann T, Leisten I, Binder G, Begemann M, Spengler S. Disturbed methylation at multiple imprinted loci: an increasing observation in imprinting disorders. *Epigenomics*. 2011; 3(5):625–637. doi:10.2217/epi.11.84. [PubMed: 22126250]
- Eggermann T, Schonherr N, Meyer E, Obermann C, Mavany M, Eggermann K, Ranke MB, Wollmann HA. Epigenetic mutations in 11p15 in Silver-Russell syndrome are restricted to the telomeric imprinting domain. *J Med Genet*. 2006; 43(7):615–616. doi:10.1136/jmg.2005.038687. [PubMed: 16236811]
- Falcon S, Gentleman R. Using GOSTats to test gene lists for GO term association. *Bioinformatics*. 2007; 23(2):257–258. doi:10.1093/bioinformatics/btl567. [PubMed: 17098774]
- Giardine B, Riemer C, Hardison RC, Burhans R, Elnitski L, Shah P, Zhang Y, Blankenberg D, Albert I, Taylor J, Miller W, Kent WJ, Nekrutenko A. Galaxy: a platform for interactive large-scale genome analysis. *Genome research*. 2005; 15(10):1451–1455. doi:10.1101/gr.4086505. [PubMed: 16169926]
- Gicquel C, Rossignol S, Cabrol S, Houang M, Steunou V, Barbu V, Danton F, Thibaud N, LeMerrer M, Burglen L, Bertrand AM, Netchine I, Le Bouc Y. Epimutation of the telomeric imprinting center region on chromosome 11p15 in Silver-Russell syndrome. *Nature Genetics*. 2005; 37(9):1003–1007. [PubMed: 16086014]
- Girardot M, Feil R, Lleres D. Epigenetic deregulation of genomic imprinting in humans: causal mechanisms and clinical implications. *Epigenomics*. 2013; 5(6):715–728. doi:10.2217/epi.13.66. [PubMed: 24283884]
- Gomes MV, Toffoli LV, Arruda DW, Soldera LM, Pelosi GG, Neves-Souza RD, Freitas ER, Castro DT, Marquez AS. Age-related changes in the global DNA methylation profile of leukocytes are linked to nutrition but are not associated with the MTHFR C677T genotype or to functional capacities. *PloS one*. 2012; 7(12):e52570. doi:10.1371/journal.pone.0052570. [PubMed: 23285094]
- Gutierrez GM, Kong E, Sabbagh Y, Brown NE, Lee JS, Demay MB, Thomas DM, Hinds PW. Impaired bone development and increased mesenchymal progenitor cells in calvaria of RB1^{-/-} mice. *Proceedings of the National Academy of Sciences of the United States of America*. 2008; 105(47):18402–18407. doi:10.1073/pnas.0805925105. [PubMed: 19020086]
- Hannula-Jouppi K, Muurinen M, Lipsanen-Nyman M, Reinius LE, Ezer S, Greco D, Kere J. Differentially methylated regions in maternal and paternal uniparental disomy for chromosome 7. *Epigenetics : official journal of the DNA Methylation Society*. 2013; 9(3)
- Harbour JW, Lai SL, Whang-Peng J, Gazdar AF, Minna JD, Kaye FJ. Abnormalities in structure and expression of the human retinoblastoma gene in SCLC. *Science*. 1988; 241(4863):353–357. [PubMed: 2838909]
- Hoglund P, Holmberg C, de la Chapelle A, Kere J. Paternal isodisomy for chromosome 7 is compatible with normal growth and development in a patient with congenital chloride diarrhea. *American journal of human genetics*. 1994; 55(4):747–752. [PubMed: 7942853]

- Horowitz JM, Park SH, Bogenmann E, Cheng JC, Yandell DW, Kaye FJ, Minna JD, Dryja TP, Weinberg RA. Frequent inactivation of the retinoblastoma anti-oncogene is restricted to a subset of human tumor cells. *Proceedings of the National Academy of Sciences of the United States of America*. 1990; 87(7):2775–2779. [PubMed: 2181449]
- Ishida M, Moore GE. The role of imprinted genes in humans. *Molecular aspects of medicine*. 2013; 34(4):826–840. doi:10.1016/j.mam.2012.06.009. [PubMed: 22771538]
- Jones PA. Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nat Rev Genet*. 2012; 13(7):484–492. doi:10.1038/nrg3230. [PubMed: 22641018]
- Kanber D, Berulava T, Ammerpohl O, Mitter D, Richter J, Siebert R, Horsthemke B, Lohmann D, Buiting K. The human retinoblastoma gene is imprinted. *PLoS genetics*. 2009; 5(12):e1000790. doi:10.1371/journal.pgen.1000790. [PubMed: 20041224]
- Kannenberg K, Urban C, Binder G. Increased incidence of aberrant DNA methylation within diverse imprinted gene loci outside of IGF2/H19 in Silver-Russell syndrome. *Clin Genet*. 2012; 81(4):366–377. doi:10.1111/j.1399-0004.2012.01844.x. [PubMed: 22248018]
- Kin T, Ono Y. Idiographica: a general-purpose web application to build idiograms on-demand for human, mouse and rat. *Bioinformatics*. 2007; 23(21):2945–2946. doi:10.1093/bioinformatics/btm455. [PubMed: 17893084]
- Koboldt DC, Steinberg KM, Larson DE, Wilson RK, Mardis ER. The next-generation sequencing revolution and its impact on genomics. *Cell*. 2013; 155(1):27–38. doi:10.1016/j.cell.2013.09.006. [PubMed: 24074859]
- Lee DH, Singh P, Tsark WM, Szabo PE. Complete biallelic insulation at the H19/Igf2 imprinting control region position results in fetal growth retardation and perinatal lethality. *PloS one*. 2010; 5(9):e12630. doi:10.1371/journal.pone.0012630. [PubMed: 20838620]
- Maksimovic J, Gordon L, Oshlack A. SWAN: Subset-quantile within array normalization for illumina infinium HumanMethylation450 BeadChips. *Genome biology*. 2012; 13(6):R44. doi:10.1186/gb-2012-13-6-r44. [PubMed: 22703947]
- Meaburn E, Schulz R. Next generation sequencing in epigenetics: insights and challenges. *Semin Cell Dev Biol*. 2012; 23(2):192–199. doi:S1084-9521(11)00198-4 [pii] 10.1016/j.semcdb.2011.10.010. [PubMed: 22027613]
- Moore T, Haig D. Genomic imprinting in mammalian development: a parental tug-of-war. *Trends Genet*. 1991; 7(2):45–49. doi:0168-9525(91)90230-N [pii] 10.1016/0168-9525(91)90230-N. [PubMed: 2035190]
- Pan Y, McCaskill CD, Thompson KH, Hicks J, Casey B, Shaffer LG, Craigen WJ. Paternal isodisomy of chromosome 7 associated with complete situs inversus and immotile cilia. *American journal of human genetics*. 1998; 62(6):1551–1555. doi:10.1086/301857. [PubMed: 9585585]
- Penaherrera MS, Weindler S, Van Allen MI, Yong SL, Metzger DL, McGillivray B, Boerkoel C, Langlois S, Robinson WP. Methylation profiling in individuals with Russell-Silver syndrome. *American journal of medical genetics Part A*. 2010; 152A(2):347–355. doi:10.1002/ajmg.a.33204. [PubMed: 20082469]
- Peng JC, Lin H. Beyond transposons: the epigenetic and somatic functions of the Piwi-piRNA mechanism. *Current opinion in cell biology*. 2013; 25(2):190–194. doi:10.1016/j.ceb.2013.01.010. [PubMed: 23465540]
- Preece MA, Abu-Amro SN, Ali Z, Abu-Amro KK, Wakeling EL, Stanier P, Moore GE. An analysis of the distribution of hetero- and isodisomic regions of chromosome 7 in five mUPD7 Silver-Russell syndrome probands. *J Med Genet*. 1999; 36(6):457–460. [PubMed: 10874633]
- Preece MA, Price SM, Davies V, Clough L, Stanier P, Trembath RC, Moore GE. Maternal uniparental disomy 7 in Silver-Russell syndrome. *J Med Genet*. 1997; 34(1):6–9. [PubMed: 9032641]
- Price S, Stanhope R, Garrett C, Preece MA, Trembath RC. The spectrum of Silver-Russell syndrome: a clinical and molecular genetic study and new diagnostic criteria. *Journal of Medical Genetics*. 1999; 36:837–842. [PubMed: 10544228]
- Proudhon C, Duffie R, Ajjan S, Cowley M, Iranzo J, Carbajosa G, Saadeh H, Holland ML, Oakey RJ, Rakyan VK, Schulz R, Bourc'his D. Protection against de novo methylation is instrumental in maintaining parent-of-origin methylation inherited from the gametes. *Mol Cell*. 2012; 47(6):909–920. doi:S1097-2765(12)00605-3 [pii] 10.1016/j.molcel.2012.07.010. [PubMed: 22902559]

- Puszyk W, Down T, Grimwade D, Chomienne C, Oakey RJ, Solomon E, Guidez F. The epigenetic regulator PLZF represses L1 retrotransposition in germ and progenitor cells. *The EMBO journal*. 2013; 32(13):1941–1952. doi:10.1038/emboj.2013.118. [PubMed: 23727884]
- Rademacher K, Schroder C, Kanber D, Klein-Hitpass L, Wallner S, Zeschnigk M, Horsthemke B. Evolutionary origin and methylation status of human intronic CpG islands that are not present in mouse. *Genome biology and evolution*. 2014 doi:10.1093/gbe/evu125.
- Reinius LE, Acevedo N, Joerink M, Pershagen G, Dahlen SE, Greco D, Soderhall C, Scheynius A, Kere J. Differential DNA methylation in purified human blood cells: implications for cell lineage and studies on disease susceptibility. *PloS one*. 2012; 7(7):e41361. doi:10.1371/journal.pone.0041361. [PubMed: 22848472]
- Sandberg AA, Bridge JA. Updates on the cytogenetics and molecular genetics of bone and soft tissue tumors: osteosarcoma and related tumors. *Cancer genetics and cytogenetics*. 2003; 145(1):1–30. [PubMed: 12885459]
- Schulz R, Woodfine K, Menheniott TR, Bourc'his D, Bestor T, Oakey RJ. WAMIDEX: a web atlas of murine genomic imprinting and differential expression. *Epigenetics : official journal of the DNA Methylation Society*. 2008; 3(2):89–96.
- Sirmaci A, Spiliopoulos M, Brancati F, Powell E, Duman D, Abrams A, Bademci G, Agolini E, Guo S, Konuk B, Kavaz A, Blanton S, Digilio MC, Dallapiccola B, Young J, Zuchner S, Tekin M. Mutations in ANKRD11 cause KBG syndrome, characterized by intellectual disability, skeletal malformations, and macrodontia. *American journal of human genetics*. 2011; 89(2):289–294. doi:10.1016/j.ajhg.2011.06.007. [PubMed: 21782149]
- Smyth, GK. *Bioinformatics and Computational Biology Solutions Using {R} and Bioconductor*. Springer; New York: 2005. Limma: linear models for microarray data.
- Spengler S, Oehl-Jaschkowitz B, Begemann M, Hennes P, Zerres K, Eggermann T. Haploinsufficiency of ANKRD11 (16q24.3) Is Not Obligarily Associated with Cognitive Impairment but Shows a Clinical Overlap with Silver-Russell Syndrome. *Molecular syndromology*. 2013; 4(5):246–249. doi:10.1159/000351765. [PubMed: 23885231]
- Staaf J, Vallon-Christersson J, Lindgren D, Juliusson G, Rosenquist R, Hoglund M, Borg A, Ringner M. Normalization of Illumina Infinium whole-genome SNP data improves copy number estimates and allelic intensity ratios. *BMC bioinformatics*. 2008; 9:409. doi:10.1186/1471-2105-9-409. [PubMed: 18831757]
- Teschendorff AE, Marabita F, Lechner M, Bartlett T, Tegner J, Gomez-Cabrero D, Beck S. A beta-mixture quantile normalization method for correcting probe design bias in Illumina Infinium 450 k DNA methylation data. *Bioinformatics*. 2013; 29(2):189–196. doi:10.1093/bioinformatics/bts680. [PubMed: 23175756]
- Touleimat N, Tost J. Complete pipeline for Infinium((R)) Human Methylation 450K BeadChip data processing using subset quantile normalization for accurate DNA methylation estimation. *Epigenomics*. 2012; 4(3):325–341. doi:10.2217/epi.12.21. [PubMed: 22690668]
- Turner CL, Mackay DM, Callaway JL, Docherty LE, Poole RL, Bullman H, Lever M, Castle BM, Kivuva EC, Turnpenny PD, Mehta SG, Mansour S, Wakeling EL, Mathew V, Madden J, Davies JH, Temple IK. Methylation analysis of 79 patients with growth restriction reveals novel patterns of methylation change at imprinted loci. *European journal of human genetics : EJHG*. 2010; 18(6):648–655. doi:10.1038/ejhg.2009.246. [PubMed: 20104244]
- Wakeling EL, Amero SA, Alders M, Blik J, Forsythe E, Kumar S, Lim DH, MacDonald F, Mackay DJ, Maher ER, Moore GE, Poole RL, Price SM, Tangeras T, Turner CL, Van Haelst MM, Willoughby C, Temple IK, Cobben JM. Epigenotype-phenotype correlations in Silver-Russell syndrome. *J Med Genet*. 2010; 47(11):760–768. doi:jmg.2010.079111 [pii] 10.1136/jmg.2010.079111. [PubMed: 20685669]
- Watanabe T, Tomizawa S, Mitsuya K, Totoki Y, Yamamoto Y, Kuramochi-Miyagawa S, Iida N, Hoki Y, Murphy PJ, Toyoda A, Gotoh K, Hiura H, Arima T, Fujiyama A, Sado T, Shibata T, Nakano T, Lin H, Ichianagi K, Soloway PD, Sasaki H. Role for piRNAs and noncoding RNA in de novo DNA methylation of the imprinted mouse Rasgrf1 locus. *Science*. 2011; 332(6031):848–852. doi:10.1126/science.1203919. [PubMed: 21566194]
- Weinberg RA. The retinoblastoma protein and cell cycle control. *Cell*. 1995; 81(3):323–330. [PubMed: 7736585]

- Willemsen MH, Fernandez BA, Bacino CA, Gerkes E, de Brouwer AP, Pfundt R, Sikkema-Raddatz B, Scherer SW, Marshall CR, Potocki L, van Bokhoven H, Kleefstra T. Identification of ANKRD11 and ZNF778 as candidate genes for autism and variable cognitive impairment in the novel 16q24.3 microdeletion syndrome. *European journal of human genetics : EJHG*. 2010; 18(4):429–435. doi: 10.1038/ejhg.2009.192. [PubMed: 19920853]
- Woodfine K, Huddleston JE, Murrell A. Quantitative analysis of DNA methylation at all human imprinted regions reveals preservation of epigenetic stability in adult somatic tissue. *Epigenetics & chromatin*. 2011; 4(1):1. doi:10.1186/1756-8935-4-1. [PubMed: 21281512]
- Yandell DW, Campbell TA, Dayton SH, Petersen R, Walton D, Little JB, McConkie-Rosell A, Buckley EG, Dryja TP. Oncogenic point mutations in the human retinoblastoma gene: their application to genetic counseling. *The New England journal of medicine*. 1989; 321(25):1689–1695. doi:10.1056/NEJM198912213212501. [PubMed: 2594029]
- Yang IV, Coldren CD, Leach SM, Seibold MA, Murphy E, Lin J, Rosen R, Neidermyer AJ, McKean DF, Groshong SD, Cool C, Cosgrove GP, Lynch DA, Brown KK, Schwarz MI, Fingerlin TE, Schwartz DA. Expression of cilium-associated genes defines novel molecular subtypes of idiopathic pulmonary fibrosis. *Thorax*. 2013; 68(12):1114–1121. doi:10.1136/thoraxjnl-2012-202943. [PubMed: 23783374]

Figure 1.

Methylation microarray analysis pipeline: (A) Each of the ~485 000 CpG assays measures methylation change for both the Methylated and Unmethylated moieties. (B) For a CpG to be considered display a significantly methylation difference, the signal for both moieties must differ from the control group with an FDR < 50%, in an inverse direction to each other, i.e., the methylated moiety increasing and the unmethylated moiety decreasing, or either the methylated or unmethylated moiety differs from the control group with FDR < 50% and the other moiety does not significantly change (FDR > 70%). (C) CpGs are scored on a per-

individual basis for each SRS patient versus the control group (see Figure 2). If the CpG is hypomethylated versus controls it receives a score of -1 , a score of $+1$ if it is hypermethylated, and a score of 0 if there is no change. The SRS group-wise CpG score is the sum of the scores across all 18 SRS individuals. (D) Neighbouring CpGs were subsequently grouped together by genomic region to assess regional methylation differences in specific genomic contexts.

Figure 2.

A,B,C. Genomic regions were scored for differences in methylation. The range of scores for controls is shown as red, and the range of scores for SRS patients is shown in blue. For each genomic region tested (CpG islands, Gene Promoter regions and Gene Body regions), the variance was greater in SRS than in controls, with no skew toward hypo- or hypermethylation. D. 174 promoter regions and 277 gene body regions showing differences in methylation in SRS were compared. 15 genes displayed methylation differences in both the gene body and the gene promoter region.

Figure 3.

Study design. Control methylome scores were taken from four different studies. Six in-house controls were analysed on the same array as the SRS patient samples, three additional sets of controls were taken from data deposited in GEO. These were from studies GSE32148 (20 controls), GSE35069 (12 controls) and GSE42865 (three controls). DNA was extracted from whole blood for each of the 18 SRS samples and for 6 controls from study GSE35069. All other control samples used DNA from only the PMBC fraction of blood. Methylation levels at each of the 485 000 probes were compared for each SRS sample individually versus the combined methylation levels of all the controls together, which represented an average 'normal' level of methylation. Thus 18 independent comparisons were made and the results from each combined (see Figure 1 for pipeline).

CpG islands differentially methylated in SRS patients versus controls

Figure 4.

Genomic locations of CpG islands that display differences in methylation in the SRS patient group. Grey squares represent hypermethylated CpG islands and grey triangles represent hypomethylated CpG islands. Each chromosome is divided in half with the left hand side showing the location of CpG islands analysed, and the right hand side illustrating gene density.

Table 1

313 CpG islands with significant methylation differences in the SRS patient group were compared with known imprinted regions. For the purpose of comparison, imprinted regions were defined as being within 100kb of an imprinted DMR, or where an imprinted DMR had not been defined for a given locus, as being within 100kb of any imprinted gene. SRS patients were assigned unique identifier numbers e.g. SRS4. For each SRS patient, if 20% or more of the probes in a CpG island exhibited hypo- or hypermethylation, this is shown in the table (“+” = hypermethylation, “-” = hypomethylation). Grey boxes indicate a known SRS associated epimutation. Summarised pre-existing clinical information is shown. (Cla = Classic, “.” = not data). Classic SRS have somewhat more severe growth restriction, and have at least two other typical features of SRS including postnatal growth restriction, relative macrocephaly, body asymmetry and typical facial features. “Mild SRS”, which is sometimes called “atypical SRS”, have growth restriction but less typical additional features or less than two additional typical characteristics.

Individual SRS patients																						
DMR coordinates (hg19)	Gene/DMR	imprinted DMR/ imprinted gene	distance from DMR	SRS 4	SRS 5	SRS 6	SRS 12	SRS 17	SRS 19	SRS 22	SRS 23	SRS 26	SRS 29	SRS 30	SRS 31	SRS 39	SRS 44	SRS 50	SRS 62	SRS 94	SRS 96	
chr7:50849752-50850871	<i>GRB10</i>	imprinted DMR	0 bp			+					+							+				
chr7:94284858-94286527	<i>PEG10</i>	imprinted DMR	0 bp			+					+							+				
chr7:130130739-130133111	<i>MEST</i>	imprinted DMR	0 bp			+					+							+				
chr11:2019565-2019863	<i>H19</i>	imprinted DMR	0 bp				-						-		-	-	-			-		
chr13:48890957-48891549	<i>RBI</i>	imprinted DMR	1087 bp			+	+	+	+				+	+		-	+	+	+		+	
chr16:89299274-89299874	<i>ANKRD11</i>	imprinted gene	24160 bp	+		+		+		+						+				+		
chr20:30160786-30161107	<i>MCTS2</i>	imprinted DMR	25494 bp			+				+					+	+	+	+		-	+	

Clinical classification	Severity	Mild	Cla	Mild	Mild	Cla	Mild	Mild	Cla	Mild	Cla	Cla	Cla	Cla	Mild	Cla	Cla	Mild	Cla	Cla	Cla
	mUPD7	N	.	Y	N	N	N	N	N	N	N	N	N	N	Y	N	N	Y	N	N	N
	Pre-study <i>H19</i> ICR1 hypomethylation diagnosis	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	Body asymmetry	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	Clinodactyly	N	.	Y	N	N	N	N	N	N	N	N	N	N	Y	N	N	Y	N	N	Y
	Genital abnormalities	N	.	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	Hypoglycaemia	N	.	Y	N	N	N	N	N	N	N	N	N	N	Y	N	N	N	N	N	N
Gender	M	M	F	F	F	M	F	F	F	M	F	M	F	M	F	M	M	M	F	M	F

Table 2

15 regions demonstrate methylation differences in SRS patients versus controls in both gene promoter and gene body regions. *MSS51* and *NRBP1* (both grey) were considered to be the most robust results because CpGs in their 1st exons did not influence both the promoter and the gene body methylation scores.

Gene Symbol	Promoter DMR coordinates	Prom Score	Body DMR coordinates	Body Score
<i>CHRNA</i>	chr2:233403063-233404477	-2.2	chr2:233404477-233410852	-3
<i>NRBP1</i>	chr2:27650867-27654703	-2.1	chr2:27651571-27662669	3
<i>GLT8D1</i>	chr3:52737166-52737693	3.3	chr3:52728804-52737578	2.7
<i>CARD6</i>	chr5:40841298-40841590	2.6	chr5:40841493-40854992	2.8
<i>GPR150</i>	chr5:94955575-94957269	-3	chr5:94956017-94957269	-4.2
<i>MSS51</i>	chr10:75193254-75194398	3	chr10:75184282-75187383	2.5
<i>SCGB1D2</i>	chr11:62008949-62009790	-2.3	chr11:62009790-62011966	-2.5
<i>LPCAT4</i>	chr15:34659304-34660076	-3.5	chr15:34657364-34659304	-4
<i>AMFR</i>	chr16:56459026-56460156	-4.4	chr16:56396075-56459217	-2.1
<i>GLTPD2</i>	chr17:4691376-4692377	-2.8	chr17:4692287-4693260	-2.3
<i>PABPC1L2B</i>	chrX:72223388-72225115	2.5	chrX:72223388-72225115	2.5
<i>GPR174</i>	chrX:78426877-78427682	2.5	chrX:78426877-78427682	2.5
<i>UTY</i>	chrY:15591533-15593045	2.5	chrY:15451782-15591617	3.3
<i>SRY</i>	chrY:2654978-2655977	2.5	chrY:2654978-2655740	2.5
<i>RPS4Y1</i>	chrY:2708972-2709627	2.8	chrY:2709627-2718782	3