Use of multiplex ligation-dependent probe amplification increases the detection rate for 11p15 epigenetic alterations in Silver–Russell syndrome

Silver–Russell syndrome (SRS) describes a malformation syndrome with severe intrauterine and postnatal growth retardation. Currently, two major (epi)mutations have been described: while approximately 10% of patients carry a maternal uniparental disomy of chromosome 7 (UPD7), 35–60% show a hypomethylation at the $H19$ differentially methylated regions (DMRs) in 11p15. Until recently, a Southern-blot based test was routinely used to identify epimutation carriers. Nevertheless, this test was time consuming and hampered by the huge amount of genomic DNA needed. With the methylation-specific multiplex ligation-dependent probe amplification assay (MLPA) for SRS, a PCR-based test is now available, allowing the analysis also of small amounts of DNA. Probes in this assay hybridize to the $H19$ DMRs but do not cover the genomic target of the Southern-blot probe. We now screened 72 patients with SRS by MLPA. Hypomethylation of the $H19$ DMRs was confirmed in all patients analyzed by Southern blot. In addition, we identified six individuals with hypomethylation of the $H19$ DMR who had previously normal blot results. This discrepancy can be explained by the observed generally lower degree of demethylation in this group, possibly not detectable by the less sensitive Southern-blot method but also with a varying degree of methylation at different DMRs in the same individual. Apart from hypomethylation in the $H19$ DMR, we observed a slight demethylation for one of the $IGF2$ probes. The total detection rate of 11p15 hypomethylation is now increased to >38%. Considering maternal UPD7 and chromosomal aberrations, (epi)genetic alterations now account for more than 50% of SRS patients. In summary, MLPA represents an easy, low cost and reliable system in the molecular diagnostics of SRS.

Silver–Russell syndrome (SRS or RSS; OMIM 180860) describes a malformation syndrome. In addition to the obligatory pre- and postnatal growth restriction (<P3), SRS includes a relative macrocephaly, a small triangular face, asymmetry, clinodactyly V and other less constant features. A clinical scoring system to assist the clinical diagnosis has not yet been established; thus, it is influenced by the experience of the clinical investigator. Furthermore, the clinical picture of SRS in adulthood is less clear than in early childhood. Therefore, a reliable molecular tool is needed to confirm the clinical diagnosis of SRS. The identification of different genetic alterations in SRS patients indicate that imprinted regions on at least two different chromosomes are involved in...
the aetiology of the disease, while maternal uniparental disomy (UPD) of chromosome 7 is a well-known disturbance accounting for nearly 10% of patients (1). (epi)genetic mutations in 11p15 have recently been described as a major genetic disturbance in SRS patients (2–4). Mutations in 11p15 mainly comprise hypomethylation of the imprinting centre region 1 (ICR1), in single cases duplications of maternal 11p15 material have been reported (5). Interestingly, opposite (epi)mutations are involved in the overgrowth disease Beckwith–Wiedemann syndrome (BWS). Thus, SRS and BWS can be regarded as two genetically and clinically opposite clinical pictures. Furthermore, SRS is the first human disorder associated with epigenetic mutations, affecting two different chromosomes.

The 11p15 imprinting cluster contains a number of imprinted genes whose expression is regulated by two different imprinting centres (ICR1 and ICR2). The telomeric ICR1 confers a differential chromatin architecture to the two parental alleles, leading to reciprocal expression of IGF2 and H19 (Fig. 1). The paternally expressed IGF2 is a single chain polypeptide and is involved in development and growth. The H19 gene is expressed from the maternal allele and encodes a 2.3-kb non-coding mRNA, which is strongly expressed during embryogenesis. The physiological function of H19 is currently unknown. IGF2 and IGF2 are coexpressed in endoderm- and mesoderm-derived tissues during embryonic development. The IGF2/H19 region includes several differentially methylated regions (DMRs), two of which are paternally methylated [Fig. 1; (6, 7)]. The ICR1 contains seven CTCF target sites (CTCF1–CTCF7) in the DMR 2-kb upstream of H19 and shows allele-specific methylation. The zinc-finger binding factor CTCF binds to the maternal unmethylated ICR1 copy and thereby forms a chromatin boundary. This CTCF-binding mechanism blocks IGF2 and promotes H19 transcription of the maternal 11p15 copy.

Until recently, molecular genetic testing for detection of ICR1 hypomethylation routinely consisted of a methylation-sensitive Southern-blot analysis (2, 4). The respective probe hybridizes close to the third CTCF-binding site (Fig. 1). However, this method is hampered by the general disadvantages of Southern blotting that a huge amount of DNA (~10 μg) is necessary, making an analysis of cases where only small amounts of DNA are available difficult. Therefore, the development of a multiplex ligation-dependent probe amplification (MLPA) is very helpful to circumvent this problem. MLPA is a PCR-based hybridization technique in which multiple DNA sequences are amplified using a single primer pair, allowing accurate quantification of each individual target sequence. In contrast to Southern blotting, only 100–200 ng of genomic DNA are necessary.

The purpose of this paper is to estimate the strength of MLPA as a routine method for SRS diagnosis and to get further insights in 11p15 alterations.

**Patients**

The studied population consisted of 72 patients with SRS, ascertained as part of ongoing molecular investigations on SRS (4, 8).

The diagnosis of SRS was based on the following criteria: intrauterine growth retardation (birth weight or length below the third percentile), lack of postnatal catch-up growth, and at least two of the following criteria: typical face, relative macrocephaly, and skeletal asymmetry. In all patients, maternal UPD7 had been investigated earlier (8, 9). Forty-three of these patients were screened for hypomethylation of the CTCF3 in the ICR1 by methylation-specific Southern blot of an HpaII restriction site before (4).
To further validate the sensitivity of the test, we screened 20 patients with isolated intrauterine and postnatal growth retardation as well as 15 healthy German controls. As positive controls, we used DNA samples of two patients with duplications in 11p15 (4, 10) and three BWS patients carrying a KCNQ1 hypomethylation and ICR1 hypermethylation, respectively.

The study was approved by the Ethics Committee of the University Hospital Aachen.

Materials and methods

Genomic DNA was extracted from peripheral blood by a simple salting out procedure.

MLPA analysis was carried out according to the manufacturer's recommendations (MRC Holland, Amsterdam/NL). The principle of methylation-sensitive MLPA has been previously described by Nygren et al. (11). Briefly, 200 ng DNA was denatured and hybridized overnight at 60 °C with the SALSA probe mix, ME030BWS/RSS. This mix contains 45 probes, among them 27 specific for the BWS/SRS region in 11p15. The MLPA assay allows the detection of imbalanced copy numbers for the different genes as well as abnormal methylation, 13 of the 11p15 probes are methylation-specific as they contain a HhaI recognition site. In detail, seven probes (five of them methylation-specific) cover the HhaI gene and its differentially methylated 5' region (Fig. 1). In case of IGF2, three out of four probes harbour an HhaI recognition site and are localized in the DMR0 (Fig. 1). Further, 14 oligonucleotides hybridize to the KCNQ1 gene and the included DMRs (five methylation specific).

After ligation only and ligation with HhaI restriction, PCR amplification was carried out with the specific SALSA FAM PCR primers. One microlitre of diluted PCR products (1:3) were mixed with 0.5 µl size standard and 8.5 µl deionized formamide. Amplification products were run on an AB3130 automatic sequencing system equipped with a 36 cm capillary using POP7 as polymer.

Electrophoretic data were analyzed with an appropriate Genemapper file. Calculation of copy numbers and degree of methylation was performed using a modified excel spreadsheet originally developed for BRCA2 MLPA analysis from the Clinical Molecular Genetics Laboratory, University Hospital Leeds/UK (http://leedsdna.info/) (Fig. 2).

As the probe H19-N0213-L9999 covers a frequent single nucleotide polymorphism (SNP) (rs10732516) within CTCF6 and affects the respective HhaI restriction site, we directly sequenced this region to estimate the influence of the SNPs on the hybridization efficiency of the probe. Sequencing was performed according to standard procedures in a number of patients.

Results

The robustness of the MLPA test in regard to copy number determination was confirmed by analysis of two patients with 11p15 duplications, among them one with a small 1 Mb duplication restricted to the ICR2 (10). For the 15 control samples, the following hybridization ranges of the H19 methylation-specific probes were obtained: probe N0213-L9999: 0.39 ± 0.09, probe 6264-L5774: 0.47 ± 0.03, probe 6265-L5771: 0.48 ± 0.02, probe 6266-L5772: 0.50 ± 0.09, and probe 7177-L6786: 0.49 ± 0.09.

In the studied population, neither duplications nor deletions were observed.

In our SRS cohort (n = 63) without maternal UPD7, we identified hypomethylation of the ICR1 in 44.4% (28/63) by MLPA. This rate is remarkably higher than the frequency determined by Southern blotting: out of the 63 patients, 43 cases had been tested by Southern-blot analysis before (4), in 37.2% (16/43), a hypomethylation at the HpaII site close to the CTCF3 was detected. MLPA testing of these patients confirmed hypomethylation in 16 cases studied before, but in further six cases, ICR1 hypomethylation became obvious despite the initially negative results from the CTCF3 testing, corresponding to a frequency of nearly 51.2% (22/43) in this group.

A more detailed comparison of the methylation patterns between those patients with normal blot results but hypomethylation in MLPA (n = 6) and those with demethylation detectable both in blot analysis and in MLPA (n = 16) indicated that the first group showed a generally milder hypomethylation (Fig. 3).

Considering our total population of patients with SRS (n = 72), a frequency of 38.8% (28/72) of the ICR1 hypomethylation and 12.5% (9/72) of the maternal UPD7 could be determined. Corresponding to previous reports (12, 13), our 11p15 epimutation carriers have a more severe phenotype (foetal growth retardation, relative macrocephaly, prominent forehead and body asymmetry) than those patients without recognizable genetic disturbances, while the patients with maternal UPD7 showed a milder clinical picture (G. Binder, in preparation).

In the 20 patients with isolated intrauterine growth restriction postnatal growth restriction
methylation was detected (Fig. 3). While four of the five H19-specific probes gave reliable results and allowed a clearcut differentiation between hypomethylation and normal methylation, analysis of the probe N0123-L9999 hybridizing to the sixth CTCF-binding site showed ambiguous results (Fig. 3). By sequencing six DNA samples with methylation ratios of this probe deviating from the results of the other four H19 fragments, we confirmed hetero- or homozygosity of that allele of the frequent SNP rs10732516 that hampers HhaI digestion and thus leads to an altered hybridization of N0123-L9999. We therefore excluded the results obtained by this probe from further considerations.

While analysis of the control and IUGR samples without hypomethylation showed a constant methylation ratio for the four different H19 specific methylation probes (Fig. 3), typing results of the patients with SRS with ICR1 hypomethylation revealed a broad range of demethylation, corresponding to the mosaic distribution of the epimutation in the patients (2).

For the IGF2-specific methylation probe, 7174-L6783, no methylation changes were found in patients with SRS and controls, whereas with probe IGF2 6269-L5775, a broad variation in methylation was observed in patients with SRS but also in controls. Interestingly, patients with H19 hypomethylation also carried a slight hypomethylation at the IGF2 probe locus 7175-L6784 in the DMR0.

Apart from the BWS samples used for validation of the assay (Fig. 2), hybridization of KCNQ1-specific probes did not reveal any abnormality in our cohort.

Fig. 2. Examples of excel spreadsheet plots for the methylation-specific run. (a) Plot of a control sample: dosage probes (light grey) show a normal copy number; the three gaps for the methylation-sensitive control probes confirm that the HhaI digest is complete. Methylation-specific probes (dark grey) for H19, IGF2 and KCNQ1 reveal normal patterns. (b) Plot of Beckwith–Wiedemann syndrome (BWS) DNA sample with a KCNQ1 hypomethylation: all the KvDMR probes give reduced signals. (c) Plot of a Silver–Russell syndrome (SRS) DNA sample with hypomethylation indicated by reduced hybridization of the H19 (and IGF2) probes. Note that some probes show an apparently abnormal hybridization pattern in case of the BWS and the SRS samples, which can be explained by polymorphic sites within the hybridization region (H19DMR N0213-L9999) or a generally broader methylation pattern.

and in the healthy controls, no case of altered methylation was detected (Fig. 3).

Detailed analysis of the hybridization patterns of the H19 and the IGF2 methylation-specific probes demonstrated that their suitability to detect aberrant methylation is different (Fig. 3).
Discussion

By testing a large cohort of SRS patients, we demonstrated that the recently developed methylation-specific MLPA approach to detect (epi)genetic disturbances in 11p15 represents a sensitive and reliable tool in molecular diagnosis of SRS. While the conventional technique for ICR1 methylation consists of Southern blotting analysis of a HpaII site close to the CTCF3 and requires a huge amount of genomic DNA, MLPA needs only one fiftieth of DNA and is less time consuming.

The exclusion of copy number variations by MLPA in the 11p15-imprinted region indicates that duplications in SRS are rare. Microdeletions in the H19 DMR as reported in BWS patients (14) can be excluded in our SRS cohort. Furthermore, an aberrant methylation for the KCNQ1 locus was also not detected, thus confirming the observations that epimutations in the ICR2 do not contribute to the SRS phenotype (2, 4).

As we could show that the MLPA approach is much more sensitive than the Southern blotting procedure restricted to the HpaII restriction site at CTCF3, we identified six patients with a hypomethylation in MLPA but normal methylation patterns at the third CTCF. This finding confirms a recent study of Zeschnigk et al. (submitted) who demonstrated that their real-time PCR-based methylation assay of the CTCF6 as well as the commercially available MLPA assay have a higher sensitivity than the Southern-blot analysis of the HpaII restriction site close to the CTCF3. Several causes for this discrepancy can be presumed from the technical point of view, the blotting procedure might be less sensitive and might thereby miss detection of cases with only a slight hypomethylation. This assumption is supported by our finding that those cases with discrepant results in blotting and MLPA show a generally milder demethylation than those with concordant results (Fig. 3). The discrepancy might, furthermore, be
explained by a general variation in DNA methylation across individuals in the same locus, which is attributable to heritable and environmental factors (15).

According to Takai et al. (16), the ICR1 CTCF6 is the key regulatory domain for switching between H19 and IGF2 expression. Hypomethylation of the sixth CTCF-binding site can therefore be regarded as the most specific genetic marker for molecular SRS testing. Unfortunately, the reliability of the CTCF6 specific MLPA probe, N0213-L9999, is significantly reduced because the respective HhaI recognition site is affected by a frequent SNP. Therefore, we decided to exclude the MLPA results of this locus from interpretation despite the central role of the sixth CTCF-binding site. Nevertheless, our results demonstrate that reliable results can also be obtained if we restrict our calculations to the other methylation-specific H19 probes.

Interestingly, one of the methylation-specific MLPA probes for the DMR0 of IGF2 (7175-L6784) reveals a slight hypomethylation in patients with SRS with a H19 DMR hypomethylation. However, from the diagnostic point of view, the relevance of the probes hybridizing to the IGF2 DMR0 in the current MLPA assay version is questionable, and the inclusion of IGF2 DMR2-specific probes could be helpful to find out more about this.

Based on our results, we think that MLPA analysis presents a reliable, fast and high throughput system for performing SRS testing and significantly increases the detection rate for 11p15 hypomethylation. In our opinion, it should be the basic technique in the molecular diagnosis of SRS, in case of negative findings testing for maternal UPD7 and chromosomal aberrations should follow. In this context, the inclusion of methylation-specific probes hybridizing to imprinted region on chromosome 7 in a SRS specific MLPA assay will be helpful. With this diagnostic algorithm, a total detection rate of more than 50% can be achieved.

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References