npg European Journal of Human Genetics (2008) 16, 328–334
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www.nature.com/ejhg

ARTICLE

IGF2/H19 hypomethylation in Silver–Russell syndrome and isolated hemihypoplasia

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Silver–Russell syndrome (SRS) is a clinically and genetically heterogeneous syndrome characterized by severe pre and postnatal growth retardation, body asymmetry and a typical facial phenotype with a triangular face and relative macrocephaly. In 30% of patients, the differentially methylated *IGF2/H19* imprinting center region (*ICR1*) on chromosome 11p15 was found to be hypomethylated, as determined by Southern blot analysis of an *Hpa*II restriction site close to the third *CTCF*-binding site (*CTS*3) within *ICR1*. Using bisulfite treatment and a real-time PCR-based methylation assay (QAMA), we analyzed the third and sixth *CTCF*-binding sites (*CTS3*, *CTS6*) in 5 patients with *CTS3* hypomethylation, in 14 patients who were suspected to have SRS but were normal by Southern blot analysis, and in 1 patient with body asymmetry without any other features of SRS or Beckwith–Wiedemann syndrome (BWS). In all 5 patients with *CTS3* hypomethylation, in 5 of 14 patients who were judged to be normal at *CTS3* by Southern blot analysis and in the patient with isolated body asymmetry, we found *CTS3* and *CTS6* hypomethylation by QAMA. Using methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA), we obtained similar results at four additional *ICR1* sites in the *CTS6* region. These results show that *ICR1* hypomethylation occurs more often in SRS patients than as previously thought as well as in isolated hemihypoplasia. Furthermore, we show that methylation analysis by QAMA and MLPA is more sensitive in detecting *ICR1* hypomethylation than Southern blot analysis of *CTS3*.

European Journal of Human Genetics (2008) **16,** 328–334; doi:10.1038/sj.ejhg.5201974; published online 9 January 2008

Keywords: Silver–Russell syndrome; isolated hemihyperplasia; methylation testing

Introduction

Silver–Russell syndrome (SRS) was independently described by Silver[1] and Russell.[2] It is a clinically and genetically heterogeneous disorder that is characterized by short stature of prenatal onset, body asymmetry and a triangular face. Although mostly sporadic, in rare familial cases SRS is transmitted in an autosomal dominant or recessive, or X-linked dominant way.[3] Diagnosis can be difficult owing to the variable presentation of phenotypes. Maternal uniparental disomy of chromosome 7 occurs in about 10% of cases and is associated with a milder phenotype.[4,5] In a previous study, hypomethylation of the differentially methylated *IGF2/H19* imprinting center region (ICR1) on chromosome 11p15 was found in

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Received 25 April 2007; revised 6 November 2007; accepted 14 November 2007; published online 9 January 2008

approximately 30% of patients,[6] using Southern blot analysis of an *Hpa*II restriction site close to *CTS3*.

The same ICR is hypermethylated in about 15% of patients with Beckwith–Wiedemann syndrome (BWS), which is characterized by pre and postnatal overgrowth.[7,8] This suggests that SRS is genetically and clinically opposite to BWS.[9] However, in 60% of cases, BWS is caused by hypomethylation of a second differentially methylated region at 11p15 (*ICR2*), which regulates expression of the transcript *KCNQ1OT1*.[10,11] This region has not been found to be epigenetically altered in patients with SRS.

The *ICR1* is flanked by two oppositely imprinted genes, *IGF2* and *H19*. The expression of these genes is controlled by competition of their promoters for transcription activation from shared enhancers located downstream of the *H19* gene. The unmethylated, maternally derived *ICR1* is bound by the zinc finger-binding factor CTCF, thus acting as a chromatin boundary. This blocks the interaction of *IGF2* with the enhancer and promotes *H19* transcription. On the paternal allele, the *ICR1* is methylated and CTCF-binding and enhancer-blocking activity is abolished, thereby allowing expression of *IGF2*.

The *ICR1* encompasses seven potential CTCF-binding sites (CTSs) that show allele-specific methylation.[12] Based on the observation that only *CTS6* methylation is correlated with the expression of either *IGF2* and *H19* in normal human embryonic tissue, a key regulatory role for *CTS6* was proposed by Takai *et al*.[13]

In previous studies on patients with SRS, methylation patterns were determined at the *H19* promoter or a region close to *CTS3* within the *ICR1*[6,14,15] by Southern blot analysis using methylation-sensitive restriction enzymes *Sma*I and *Hpa*II, respectively. Here, we have analyzed the degree of methylation of the *Hpa*II site close to *CTS3* and *CTS6* by quantitative analysis of methylated alleles (QAMA), which is a bisulfite-based real-time PCR methylation assay,[16] and of various loci located from 300 bp upstream to 1800 bp downstream of *CTS6* by multiplex ligation-dependent probe amplification (MLPA).

Materials and methods

Patient information

Patient Esr1 is the second child of a healthy non-consanguineous Turkish couple. The mother was 22 and the father was 29 years of age at the birth of the patient. Pregnancy was normal, and the girl was born at 42 weeks of gestation by cesarean section. Birth measurements were normal (Table 2). Body asymmetry was noted after birth with the left arm shorter and thinner than the right one. Later, a shorter left leg and foot were observed. The girl has 2/3–syndactyly of the toes, like her father. Motor and speech development are normal. Body measurements were low but in normal range at the age of $2\frac{1}{4}$ years (length 85 cm (10th centile), weight 11.5 kg (25th centile), OFC 46 cm (10th centile)). Further 19 SRS patients were comprised in this study. They all showed severe intrauterine and postnatal growth retardation (<3rd percentile) and at least three further signs typical for SRS according to Price *et al*.[5] All 17 patients indicated by 'AC' in the patient ID are a subset of a group of 51 patients previously described by Eggermann *et al*.[6]

DNA isolation from blood and sperm

DNAs were extracted and purified from blood and sperm cells using the FlexiGene DNA Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Semen samples were obtained from patient Esr3 and from two normal volunteers whose ejaculate was considered to be normal on the basis of WHO criteria. We used a swim-up technique for sperm separation as described elsewhere.[17] During the incubation, the motile sperm migrated into the medium, whereas the immotile and sluggish sperm and the particulate matter of semen remained in the pellet. After the incubation period of 1 h, the medium, which was enriched with motile sperm, was withdrawn and the sperm cells were used for DNA isolation. Semen sample of patient Esr3 was within the normal range with respect to sperm number (38.5×10^6/ml) and all other tested parameters such as motility and morphology.

Methylation analysis

Southern blot analysis

Southern blot analysis was performed as described previously.[6,14]

Quantitative analysis of methylated alleles

Bisulfite treatment: the procedure was modified from established protocols.[18] Genomic DNA (1 μg in 30 μl) was denatured by adding 3 μl freshly prepared 3 M NaOH and incubating the solution at 37°C for 15 min. For complete denaturation, the samples were incubated at 95°C for 1 min and immediately cooled on ice. The bisulfite solution was prepared by dissolving 8.5 g of sodium bisulfite in 15 ml degassed water, adding 1 ml of a 40 mM hydroquinone solution and adjusting the pH to 5.1 with 1000 μl of 10 M NaOH. The bisulfite solution (0.5 ml) was added to the denatured DNA, mixed and incubated at 50°C for 16 h in the dark. The DNA was recovered by using the Wizard DNA Clean-Up System (Promega) followed by elution in 100 μl water. Subsequently, 11 μl of 3 M NaOH was added and the samples were incubated for 15 min at 37°C. The solution was then neutralized by adding 110 μl of 6 M NH_4Oac, pH 7.0. The DNA was ethanol precipitated, washed in 70% ethanol, dried and resuspended in 20 μl water.

Rationale and methodological details of the real-time PCR-based QAMA assay are described elsewhere.[16] In brief, PCR was performed using a 96-well optical tray with caps at a final reaction volume of 20 μl. Samples contained 10 μl of

TaqMan® Universal PCR Master Mix, No AmpErase® UNG (uracil–N–glycosylase), 2 μl of bisulfite-treated DNA, additional 2.5 U AmpliTaq Gold (Perkin Elmer), 2.5 μM each of the primers and 150 nM each of the fluorescently labeled probes H19met and H19unmet. Initial denaturation at 95°C for 10 min to activate AmpliTaq Gold DNA Polymerase was followed by 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min (ABI Prism, 7000, Sequence Detection System). PCR primers were designed to amplify the bisulfite converted antisense strand sequence at *CTS3* and *CTS6* lacking any known nucleotide polymorphisms. Degree of methylation is measured by TaqMan minor grove binder (MGB) probes that bind to their respective target sequence. Therefore, the amplification of the methylated and unmethylated alleles is monitored independently in the same tube. The binding site of the *CTS3* and *CTS6* MGB probes cover 1 and 3 CpG dinucleotides, respectively. We used VIC-labeled MGB probes that specifically hybridize to the sequence from the unmethylated allele and an FAM-labeled probe that binds to the sequence generated from the methylated allele. The improved sequence specificity inherent to the MGB technology, which is widely used to discriminate single-nucleotide polymorphisms (SNP), allows reliable discrimination even of single base pair changes.[19,20] The cycle number at which the fluorescence signal crosses a detection threshold is referred to as C_T. The difference of both C_T values within a sample (ΔC_T) is calculated ($\Delta C_T = C_{T-FAM} - C_{T-VIC}$) and is a measure for the degree of methylation. All samples were measured in duplicate using the mean for further analysis. The relative prevalence of either the methylated or the unmethylated allele was set to 100% in case only one fluorescence signal crossed the threshold, indicating a relative absence of the opposite target. For precise quantification, a standard curve consisting of bisulfite-treated, defined mixtures of *Sss*I-methylated and unmethylated PCR products (*CTS6*) and *Sss*I-methylated and unmethylated genomic DNA (*CTS3*) is implemented in every run.[16]

QAMA primer and probes:
H19.3fw: 5′-CATAACACATAAATATTTCTAAAAACTTCTCCTTC-3′ (7872–7906); H19.3rev: 5′-GGGTTGTGATGTGTGAGTTTGTATTG-3′ (7972–7997);
H19unmet: 5′-VIC-AATTATAAAATCAAAAATAACCACA-MGB-3′ (7941–7965); H19met: 5′-6FAM-AAATCGAAAATAACCGCG-MGB-3′ (7948–7965).

H19CTS3fw:5′-cttgcttcctggcacgagGGGTTTTTGGTAGGTATAGAAATTG-3′ (5592–5617), H19CTS3rev: 5′-caggaaacagctatgacCCCCATCCAAAAAAAACTTAAAC-3′ (5749–5771), H19CTS3unmet: 5′-VIC-TTTATTATTTGGATGGTATAGAAT-MGB-3′ (5665–5688), H19CTS3met: 6FAM-TTATTATTCGGATGGTATAGAAT-MGB-3′ (5666–5687). Methylation-discriminating nucleotides in probes are underlined. Uncapitalized nucleotides do not bind to the target sequence but provide a tag that has been shown to improve amplification and facilitate sequence analysis of PCR products. Primers used for *CTS6* amplification and sequence analysis are as follows: H19nDNAfw 5′- CCATATCGGGCTACGTGTCT-3′ (7696–7715); H19nDNArev, 5′- GGGCTGTCCTTAGACGGAGT-3′ (8273–8292). All numbering refers to AF125283.

Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA)

The SALSA ME030 (Lot 0107) MS-MLPA mix was purchased from MRC-Holland (http://www.mlpa.com). The probe mix included in this kit contains 27 probes specific for the BWS/SRS chromosome 11p15 region. Of these, 13 are MS-MLPA probes, containing a *Hha*I recognition site specific to the differentially methylated regions *H19/IGF2 ICR1* and *KvDMR*, as well as several other sequences. In addition, 15 MLPA probes located outside the BWS-SRS locus are added as control probes for copy-number quantification, as well as three MS-MLPA control probes for complete *Hha*I digestion in the MS-MLPA reaction. We used a total of 400 ng of genomic DNA for each sample tested. After 16 h of hybridization at 60°C, samples were split equally into two aliquots. The first aliquot underwent ligation only, whereas the second underwent ligation plus enzymatic digestion. The ligation, enzymatic digestion and PCR amplification were performed according to the manufacturer's instructions. PCR products (1 μl) from each tube were mixed with 1 μl of internal size standard and 20 μl of deionized formamide and were injected into an ABI-3100 genetic analyzer (Applied Biosystems) equipped with a 50-cm, 16-capillary array.

The Genescan project was imported into a Genotyper file containing an appropriate table of categories and two macros for peak calling and data tabulation. Analysis of data was performed using a modified Excel spreadsheet, which contained algorithms originally created for BRCA2 MLPA analysis,and was obtained from the Clinical Molecular Genetics Laboratory, Regional Clinical Genetics Service at St James's University Hospital in Leeds, UK (http://leedsdna.info/). Data sets for both the test samples and the average of the normal control samples are imported, corrected and internally adjusted in the raw data worksheet, and dosage ratios for the probes (equivalent to dosage quotients) are calculated and listed in the analysis results section along with a probability value (for details, see http://leedsdna.info/science/dosage/REX-MLPA/REX-MLPA_analysis_User_Guide.pdf).

Results

Patient Esr1 was referred to us for BWS testing. She had body asymmetry but no sign of overgrowth, omphalocele or macroglossia (Table 2). Using a quantitative real-time PCR-based assay (QAMA),[16] which determines the methylation status of three CpG dinucleotides at *CTS6*, we observed ICR1 hypomethylation. This finding pointed to

SRS rather than BWS. *CTS3* methylation by Southern blot analysis was judged to be normal. To analyze whether these results reflected different methylation patterns between *CTS3* and *CTS6* or might be due to false interpretation of the results of Southern blot analysis, we determined the degree of methylation of the *Hpa*II site close to *CTS3* by QAMA. We found similar degrees of methylation of the two CTCF-binding sites, although *CTS3* hypomethylation was slightly less pronounced than *CTS6* hypomethylation. This suggested that methylation analysis by QAMA might be more sensitive in detecting *ICR1* hypomethylation than Southern blot analysis of *CTS3*.

Therefore, we used QAMA to analyze *CTS3* and *CTS6* methylation in five patients with SRS showing hypomethylation of the *Hpa*II site close to *CTS3* by Southern blot analysis and another 14 patients suspected to have SRS but who were judged to be normal by Southern blot analysis. In all five patients with *CTS3* hypomethylation by Southern blot analysis, we observed hypomethylation at *CTS3* and *CTS6*. Among the 14 patients who were normal at *CTS3* by Southern blot analysis, we found *CTS3/CTS6* hypomethylation in five patients (Table 1). The degree of methylation in patients with hypomethylation varied from 1 to 16% and from 2 to 22% at *CTS6* and *CTS3*, respectively (Table 1). This is significantly lower than the methylation observed in a panel of 50 DNA samples from normal individuals, which was 35 to 55% at *CTS6*. *CTS3* methylation ranged from 38 to 50% in 13 normal individuals (data not shown).

In a study by Tost *et al*,[21,22] *H19* methylation analysis using bisulfite sequencing was significantly impaired by a frequent SNP affecting a primer-binding site. To exclude this possibility in our study, we sequenced 558 bp around *CTS6* in PCR products obtained from genomic DNA of all patients showing discordant results in QAMA and Southern blot analysis and could not detect any SNPs that might affect probe or primer binding.

To confirm the methylation results by an independent method, we applied the MLPA ME030 BWS/RSS probe mix to the SRS samples. This probe mix encompasses probe sets to determine the methylation status of five *ICR1* loci located between 300 bp upstream and 1800 bp downstream of *CTS6* (Figure 1). Here, we ignore the results obtained with probe set N0213-L9999 (covering *CTS6*) as we found that probe binding is frequently impaired by SNP rs10732516. The remaining methylation-sensitive *H19* probe sets showed, within measure accuracy, similar degrees of methylation as determined by QAMA (Table 1). Hypomethylation at the *IGF2* locus (7175-L6784) was less pronounced in patients showing hypomethylation at *CTS6* (Table 1).

As transmission of SRS has been known to occur, we tested *ICR1* methylation in sperm DNA from patient Esr3 and two normal control individuals. In all samples, we found complete methylation of *CTS3* and *CTS6* by QAMA

Table 1 Results of methylation analysis

	Southern blot	*QAMA*			*MS-MLPA IGF2*			*MS-MLPA H19*
Patient Id	*CTS3*	*CTS3*	*CTS6*	*6264-L5770*	*6265-L5771*	*7177-L6786*	*6266-L5772*	*7175-L6784*
Blood DNA								
Esr1	Normal	22	16	3	26	4	18	35
Esr2	Hypomethylated	9	6	13	14	16	14	39
AC22	Hypomethylated	22	15	33	26	23	28	34
AC40	Unmethylated	2	<1	2	2.5	0	0	17
AC5	Hypomethylated	18	15	24	24	25	23	32
AC19	Unmethylated	ND	<1	2	2	6	0	32
AC27	Normal	20	11	25	33	25	23	25
AC28	Normal	11	4	12	12	12	12	26
AC30	Normal	15	11	27	31	34	30	30
AC100	Normal	18	11	23	19	14	24	33
Esr3	Normal	16	12	25	25	21	22	33
AC6	Normal	40	47	50	51	41	39	56
AC8	Normal	36	49	49	55	45	45	45
AC12	Normal	40	49	57	53	45	47	49
AC29	Normal	42	52	ND	ND	ND	ND	ND
AC65	Normal	45	43	52	50	46	45	47
AC84	Normal	41	35	56	40	46	46	47
AC104	Normal	40	41	56	53	49	54	50
AC112	Normal	40	40	56	49	45	43	48
AC117	Normal	40	49	44	51	45	54	51
Sperm DNA								
Esr3	ND	100	100	84	26	19	23	84
Normal control	ND	100	100	85	67	32	44	77
Normal control	ND	ND	100	87	87	48	48	78

The degree of methylation is given in %. ND = not done.

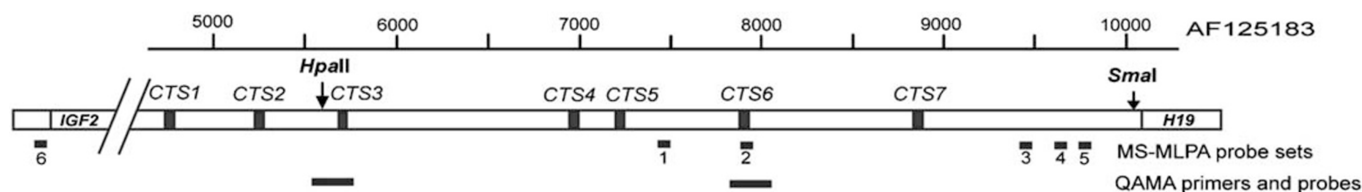

Figure 1 Map of the *H19/IGF2-ICR1*. Map positions of probes and primers used for QAMA and the probe sets used for methylation-sensitive MLPA within this region are plotted; (1) 6264-L5770; (2) N0213-L9999; (3) 6265-L5771; (4) 7177-L6786; (5) 6266-L5772; (6) 7175-L6784 MLPA probes are described in the MRC-Holland ME030 BWS/RSS probemix protocol. *Hpa*II restriction site was used for Southern blot analysis as described elsewhere.[14] Nucleotide positions are indicated in base pairs according to numbering of AF125183.

and almost complete methylation with *H19* probe set 6264-L5770 and *IGF2* probe set 7175-L6784 by MLPA. This result is consistent with a normal sperm-specific methylation pattern as published by Kerjean *et al*.[23] The remaining *H19* MLPA probe sets did not show consistent methylation patterns in six sperm DNA samples from normal controls (data not shown).

Methylation sensitive MLPA probe sets within the *KvDMR* (7171-L6780, 7172-L6781, 7173-L6782 and 6276-L5782) and *IGF2* regions (probe set 6269-L5775) revealed an unaltered methylation pattern in all patients when compared with blood DNA samples from normal controls but showed complete loss of methylation in all sperm DNAs (data not shown). The degree of methylation of the probe set *KCNQ1* (3553-L2919) was the same in all patient, sperm and normal blood samples, suggesting that this locus might lack parent-of-origin-specific methylation.

Discussion

In a recent study, Eggerman *et al* found epigenetic alterations at 11p15 by Southern blot analysis in approximately 30% of patients with an SRS phenotype.[6] In this study, the methylation status of an *Hpa*II restriction site close to *CTS3* within the differentially methylated *H19/IGF2 ICR1* was monitored. Here, we show that hypomethylation as determined by Southern blot analysis could be confirmed at *CTS3* and *CTS6* by QAMA and at additional *ICR1* loci by MLPA in all patients studied. Furthermore, using QAMA and MLPA, we were able to identify *ICR1* hypomethylation in 5 out of 14 patients (35%) that show signs typical for SRS according to Price *et al*,[5] but were judged to be normal by Southern blot analysis. On the basis of these results and the detection rate as previously determined by Eggermann *et al*,[6] we can roughly estimate that about half of all patients with a typical SRS phenotype can be detected by methylation analysis.

It is interesting to note that patient Esr1, although hypomethylated at *CTS3* and *CTS6,* does not show a typical SRS phenotype. This patient presents with prominent body asymmetry, described as hemihyperplasia, without any other features of SRS or BWS. This is distinct from the observation made in previous studies,[14,15] in which all patients with *H19* hypomethylation had pre and/or postnatal growth retardation. Hemihyperplasia is an abnormality of cell proliferation leading to asymmetric overgrowth of one or more regions of the body (OMIM, no 235 000). It is associated with an increased tumor risk and can occur isolated or as a feature of overgrowth syndromes such as BWS. In both BWS and isolated hemihyperplasia, hypermethylation of the *H19/IGF2 ICR1* was found.[24] In patient Esr1, the opposite genetic alteration, *ICR1* hypomethylation, is present. This suggests that body asymmetry in this patient is caused by growth restriction of cells of one part of the body rather than overgrowth of the opposite part. Therefore, body asymmetry in patient Esr1 is best described as hemihypoplasia, thus representing a very mild phenotype within the clinical spectrum of SRS. In some cases, it might be difficult to clinically distinguish between hemihypo and hemihyperplasia. However, the classification of patients is crucial, as an increased risk for a broad range of tumors such as Wilm's tumor has been reported for patients with hemihyperplasia.[25] In contrast, it is not to be expected that hypomethylation of the *H19/IGF2 ICR1,* as present in patients with hemihypoplasia, is associated with an increased tumor risk. But this has to be investigated in a larger group of patients with isolated body asymmetry. We recommend *ICR1* methylation testing of patients with isolated body asymmetry using QAMA or MLPA to distinguish between patients with or without increased tumor risk.

Detailed clinical data from the patients who show hypomethylation by QAMA and MLPA but not by Southern blot analysis are listed in Table 2. Among these, patient AC28 shows the lowest degree of methylation at *CTS3* and *CTS6* and the most severe pre and postnatal growth retardation, suggesting a correlation between the degree of hypomethylation and the severity of the SRS phenotype. Such a correlation has previously been observed in the studies by Gicquel *et al*[14] and Bliek *et al*,[15] using Southern blot analysis to determine *ICR1* and *H19* promoter methylation, respectively.

To analyze the possibility that the *ICR1* epimutation might be transmitted through the male germ line and thus increase the risk for SRS in offspring, we analyzed the

Table 2 Clinical data of patients who were judged to be normal at the *Hpa*II site close to *CTS3* but show *CTS6* and *CTS3* hypomethylation by QAMA

Patient id	*CTS6 methylation (%)*	Sex	*Gestational age*	*Birth weight (SDS)*	*Birth length*	*Asymmetry*	*Triangular face*	*Typical face*
Esr1	16	Female	42	3130	53	Yes	No	No
Ac27	11	Female	40	2050 (−4)	46	Yes	Yes	Yes
Ac28	4	Female	39	1380 (−5)	38	Yes	Yes	Yes
Ac30	11	Male	36	1450 (−3)	40	No	Yes	No
Ac100	11	Female	39	1960 (−3)	45	Yes	Yes	Yes
Esr3	12	Male	38	1600 (<−3)	44	Yes	Yes	Yes

degree of *CTS3* and *CTS6* methylation in spermatozoa DNA from patient Esr3. We found both sites to be fully methylated, consistent with a normal sperm-specific methylation pattern.[23,26,27] These results suggest that the epimutation is reversed in the male germ line of patient Esr3. However, it is possible that the primordial germ cells of patient Esr3, who is mosaic for the epimutation, are not affected by *ICR1* hypomethylation.

Several reasons might account for the observation that QAMA and MLPA are superior to Southern blot analysis of the *Hpa*II restriction site in SRS testing. QAMA, which is a bisulfite-based real-time PCR assay, enables precise quantification of DNA methylation. In Southern blot analysis, cases with only a slight hypomethylation might be missed. This is supported by the observation that four of the five SRS patients that were judged normal by Southern blot analysis but were hypomethylated by QAMA show a moderate degree of hypomethylation at *CTS3,* whereas three out of five patients that were diagnosed correctly by Southern blot analysis show almost complete loss of methylation. The false-negative findings by Southern blot analysis can also be caused by insufficient *Hpa*II digest incomplete DNA transfer or a high background in single lanes mimicking an altered methylation.

A prominent role of *CTS6,* as discussed by Takai *et al*,[13] is not evident in the present study, as the degree of *CTS3* and *CTS6* methylation is highly similar in all samples. MLPA probe sets located about 1.8 kb downstream of *CTS6* gave concordant results with the QAMA analysis in blood DNA of all patients, but the degree of methylation was inconsistent in sperm DNA, ranging from 32 to 87%. This suggests that the methylation imprint might be less stable in this region, at least in sperm DNA. However, except for the *CTS6* region, we cannot exclude the possibility that SNPs that are observed at high density in the *ICR1* are the cause of the observed inconsistencies at the analyzed MLPA loci. In summary, we conclude that MS-MLPA and bisulfite-based methylation analysis of *CTS3* and *CTS6* are superior to Southern blot analysis of *Hpa*II close to C*TS3* in detecting *ICR1* hypomethylation, that *ICR1* hypomethylation occurs more often in SRS patients than previously thought and that *ICR1* hypomethylation can underlie isolated hemihypoplasia.

Acknowledgements

We thank the patient and the family for their cooperation, Claudia Krallmann for the andrological evaluation of patient Esr3 and Sabine Kaya-Westerloh for technical assistance. This work was supported by DFG (Ho949/14-1).

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