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Molecular Analyses of the *BORIS* Gene in Children with Silver-Russell Syndrome

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ABSTRACT Silver-Russell syndrome (SRS) is a heterogeneous disorder associated with intrauterine and postnatal growth retardation, skeletal asymmetry and facial dysmorphisms. In 7-10% of patients maternal uniparental disomy for chromosome 7 can be observed, nearly 50% of patients carry an epimutation resulting in hypomethylation of the imprinting center region 1 (ICR1) in 11p15. This leaves 40% of patients with unknown genetic aetiology. Based on the observation that the *CTCF* homologue *BORIS* is involved in imprinted gene expression and that it binds to methylated alleles we assumed that loss-of-function mutations in *BORIS* might have similar functional consequences as an ICR1 hypomethylation. On the other hand, there is evidence that *BORIS* mutations may disturb the methylation process and therefore cause hypomethylation in the ICR1. In our study we searched for *BORIS* gene mutations in a mixed cohort of SRS patients with and without 11p15 hypomethylation to determine whether this gene is involved in SRS aetiology. Mutation analyses revealed eight genomic variants but pathogenic mutations were not observed. Thus we conclude that alterations of the *BORIS* gene are probably not associated with SRS.

INTRODUCTION

Silver Russell syndrome (SRS) (OMIM# 180860) was firstly described in 1953 by Silver and in 1954 by Russell (Silver et al. 1953; Russell 1954). The disorder is clinically variable but the major features are intrauterine and postnatal growth retardation, a characteristic triangular face with a prominent forehead, skeletal asymmetry and feeding difficulties. Maternal uniparental disomy of chromosome 7 (UPD(7)mat) is a frequent finding in SRS accounting for nearly 10% of persons (for review: Eggermann et al. 2008a). Additionally, structural chromosomal aberrations have been reported for several times in SRS affecting chromosomes 7 and 11, among them duplications of 7p11.2p-p13 and 11p15 (Abu-Amero et al. 2008).

The finding of chromosomal disturbances and altered imprinting in 11p15 in children with SRS phenotype focused the research for the etiology of SRS on this region (for review: Eggermann et al. 2008a). The 11p15 region contains two clusters of imprinted genes each of which is regulated by its own imprinting control region (ICR). The *H19* and *IGF2* (insuline growth factor-2) genes are controlled by the ICR1 while in the ICR2 the expression of *KCNQ1* and *CDKN1C* is regulated by the centromeric KvDMR1 domain (for review: Gicquel et al. 2005). In SRS, epimutations in 11p15 are restricted to the ICR1, hypomethylation in this region can be detected in approximately 50% of persons .

Numerous studies on epigenetic regulation mechanisms have identified many of the enzymes and structural proteins that interact to constitute chromatin organization and regulate gene expression. Epigenetic regulation includes post translational histone modification by silencing complexes, DNA methylation, and the formation of epigenetic chromatin by RNAs and RNA interference (Grewal and Moazed 2003; Robertson 2005). In particular DNA methylation is a potent mechanism for silencing gene expression and maintaining genome stability. A central role in gene expression in the ICR1 is mediated by the CTCF protein (CCCTC-binding factor (zinc finger protein) whose binding silences *IGF2* expression but enhances *H19* realization (Hore et al. 2008).

In 2002, Loukinov et al. reported that the CTCF protein has a family member, a sibling protein that shares the same 11ZF domain with CTCF but acts differently from CTCF. CTCF and his

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paralogue BORIS (**B**rother **O**f **R**egulator of **I**mprinted **S**ites) are DNA-binding epigenetic regulators of gene expression by influencing the nuclear architecture and transcriptional control. The two proteins show similar DNA-binding capabilities although they have probably opposite regulatory effects on gene activities. They are responsible for a parent-of-origin specific monoallelic gene expression. The ICR1 in 11p15 is methylated during spermatogenesis, specifically marking the paternally derived chromosome. CTCF binds to the unmethylated maternal ICR1 and protects this region from methylation. Additionally, CTCF blocks *IGF2* access to its enhancer and finally stimulates *H19* expression (Klenova et al. 2002; Loukinov et al. 2002; Hore et al. 2008). CTCF also negatively regulates *BORIS* expression (Renaud et al. 2007). In addition, BORIS appears to be essential for the establishment of differential methylation of the ICR1 (for review: Hore et al., 2008). Therefore we decided to screen SRS patients with ICR1 hypomethylation. We hypothesized that mutations in *BORIS* may lead to a disturbed methylation on the paternal chromosome 11 and thus result in ICR1 hypomethylation during spermatogenesis.

Nevertheless, a recent study has shown another function of BORIS: Nguyen et al. (2008) proposed that BORIS binding activity is largely independent from the methylation status of its target sequence. Furthermore, BORIS should preferentially bind to methylated DNA-binding sites because it is prevented from binding to unmethylated target sites by CTCF (Nguyen et al. 2008). We therefore assumed that mutations in *BORIS* might prevent its binding to the methylated ICR1, the functional result should be similar to that ICR1 hypomethylation which allows CTCF to bind and therefore inhibits BORIS from binding and working properly. However, Bernier- Latmani et al. (2009) did not find evidence for mutations in *BORIS* gene in 36 SRS patients and with ICR1 hypomethylation. Therefore we decided to analyze SRS patient without an ICR1 hypomethylation. It is worthy to study as there is functional evidence for its central role in establishment and regulation of the ICR1 in 11p15.

MATERIAL AND METHODS

Study Population

The study population consisted of 20 with the clinical diagnosis of SRS, 10 of these patients carried a hypomethylation in 11p15, 10 were idiopathic. Chromosomal aberrations, UPD(7)mat as well as endocrinological abnormalities were excluded before. Hypomethylation in the ICR1 on 11p15 was confirmed/excluded by MS-MLPA (Eggermann et al. 2008b). The diagnosis of SRS was based on at least three of the following criteria: intrauterine and postnatal growth retardation, a triangular face, relative macrocephaly and skeletal asymmetry. The control cohort consisted of 140 healthy German controls. The study was approved by the ethical committee of the University Hospital Aachen.

DNA Analysis

Genomic DNA was extracted from peripheral blood lymphocytes by standard techniques. All samples were preamplified by whole genome amplification (WGA) using the WGA kit provided by SigmaAldrich (Saint Louis/USA) to increase the amount of DNA.

The *BORIS* gene consists of ~28 kb and 11 exons. The genomic DNA sequence of the *BORIS* gene was taken from AceView (http://www.ncbi.nig.gov). Information on primers and PCR conditions can be obtained from Table 1. PCR was performed according to standard protocols in a 25 µl volume using recombinant Taq Polymerase (Invitrogen GmbH, Karlsruhe/Germany). Fragment sizes were between 120-550 bp. Due to its size exon 11 was divided into three overlapping fragments. PCR products were directly sequenced using the Big Dye Terminator Cycle Sequencing System (ABI, Weiterstadt/Germany). For rapid genotyping of 140 control DNA samples for the variant p.V329I in exon 5 of the *BORIS* gene, we established a pyrosequencing approach using the following primers: BORIS-Pyro-Forward 5´AAC TAT GTG ATT TTA CAG GAA CCA GGC CCT AC 3´, Reverse 5´CAT GAG TAT GTT TAT AGC GCC TGT GTC GG 3´ and for sequencing analyze BORIS-Pyro-S 5´TGT CAC CAG TGG AGA A 3´.

RESULTS AND DISCUSSION

In our total cohort of 10 SRS patients with a 11p15 hypomethylation and 10 patients without known (epi)genetic mutation we detected 8 genomic variants. Five of them have previously been reported (Table 2). We identified three so far unknown variants in exons 5 and 9 and in the 3´- untranslated region of exon 11, respectively.

In exon 5 we found heterozygosity for a G>A transition at nucleotide position g.6267 leading to a change from Val to Ile (p.V329I) in one SRS patient with ICR1 hypomethylation. By screening 140 healthy controls we failed to find the same substitution but sequencing of the patients´ healthy mother revealed heterozygosity for the same variant. Based on this finding and the fact that this amino acid residue is not evolutionary conserved we excluded the variant to be pathogenic.

In another SRS patient with 11p15 hypomethylation we identified heterozygosity for a silent substitution in exon 9 (g.16382A>G). Based on the fact that this variant is silent we concluded that it is not pathogenic.

Heterozygosity for the new variant in the 3´UTR (g.26945T>C) was detectable in an idiopathic SRS patient as well as in an 11p15 hypomethylation carrier, therefore a pathogenic relevance for these etiologically different groups is not conceivable.

In summary, we did not detect any pathogenic mutation in the *BORIS* gene similarily to the, Bernier- Latmani et al. (2009) data. Of course we can not exclude that point mutations or large genomic imbalances in *BORIS* might be responsible for single cases. Although many

Table 1: Description of PCR conditions and primer sequences for analysis of the BORIS gene.

Exon /Fragment	*Primer sequence (5'>3')*	*Annealing temperature/ Number of cycles*	*Size of exon*	*Size of PCR products*
EXON 1	F 5'- ACT ACC CAG TCT TCC AGT GC-3' R 5'- GCC TAG CAA GTT TCA GGT CC-3'	*Platinum Taq* (Invitrogen) Annealing temperature -57^{O}C Number of cycles- 35	71 bp	604 bp
EXON 2	F 5'- CAT TGT TCT GAC CCT ACT AA-3' R 5'- TGG GAA GTA TTT GTA CTG TC-3'	*Taq* polymerase (Invitrogen) Annealing temperature -53^{O}C Number of cycles- 34	553 bp	829 bp
EXON 3	F 5' - GAC AGT ACA AAT ACT TCC CA-3' R 5' - CAA TTA CCA TCA CCT GCC CA-3'	*Taq* polymerase (Invitrogen) Annealing temperature -55^{O}C Number of cycles- 34	210 bp	565 bp
EXON 4	F 5'- TGG TGT CCT GAA TTG CTT CG-3' R 5'-TAC CAC AGA TGC ATG TGA GA-3'	*Taq* polymerase (Invitrogen) Annealing temperature -51^{O}C Number of cycles- 34	170 bp	511 bp
EXON 5	F 5'-TCT CAC ATG CAT CTG TGG TA-3' R 5'-TGG AGT AAC TTG TAC AGC AG-3'	*Taq* polymerase (Invitrogen) Annealing temperature - 51^{O}C Number of cycles- 34	133 bp	333 bp
EXON 6	F 5'-CTC TCA GAA CTG CTG CTC AT-3' R 5'-AAC ACT GTA GGT ACT AGG CC-3'	*Taq* polymerase (Invitrogen) Annealing temperature - 54^{O}C Number of cycles- 34	120 bp	387 bp
EXON 7	F 5' - GAC ATA AGC TGT CTG GGC TT-3' R 5' - ATG AGA TGC AGC CAC CAG AT-3´	*Taq* polymerase (Invitrogen) Annealing temperature -59^{O}C Number of cycles- 34	149 bp	498 bp
EXON 8	F 5'-GGA AGG AAC TAG CAT GTT CC-3' R 5'-GCT CAC AGT TGC TTT CTG GT-3'	*Taq* polymerase (Invitrogen) Annealing temperature - 51^{O}C Number of cycles- 34	160 bp	424 bp
EXON 9	F 5' -GAG TGA ATG CTG CCT TGT TC-3' R 5' -TGA CAT TGT CCT CCT GAG AT-3'	*Taq* polymerase (Invitrogen) Annealing temperature - 51^{O}C Number of cycles- 34	182 bp	409 bp
EXON 10	F 5'-TAG CGA GGT GTG TGG AAT TG-3' R 5'-GAC CGA CTG GTG GAC AAA TA-3'	*Taq* polymerase (Invitrogen) Annealing temperature - 51^{O}C Number of cycles- 34	166 bp	426 bp
EXON 11.1	F 5'-AAC ATG CTT GCT GCC GTG TT-3' R 5' -TGA CTG TGG AAG CAC TAG CT-3'	*Taq* polymerase (Invitrogen) Annealing temperature - 57^{O}C Number of cycles- 34	151 bp	693 bp
EXON 11.2	F 5'-GAG GGT AAC ACC TCA AAC CT-3' R 5'-TTT CAT ACC TGC CAA GGA GC-3'	*Taq* polymerase (Invitrogen) Annealing temperature - 51^{O}C Number of cycles- 34	-	434 bp
EXON 11.3	F 5'-GGT ATA GGA GCA AGG TTC AC-3' R 5'-CTC CAC ATT CTC TCC AGA CA-3'	*Taq* polymerase (Invitrogen) Annealing temperature - 57^{O}C Number of cycles- 34	-	749 bp

Table 2: Description of genomic variants in our cohort of Silver-Russell syndrome patients (The nucleotide numbering is based on clone gi:51511747; the new variants are printed in bold face).

Exon	*Variant*	*Frequency in idio pathic SRS*	*Frequency in SRS patients with ICR1 hypome-thylation*
2			
(rs6070128)	g.1041G/G	3	4
	g.1041C/C	2	3
	g.1041G/C	5	3
(rs6025606)	g.1422A/A	2	3
	g.1422G/G	5	5
	g.1422A/G	3	2
5			
	g.6267G/G	10	9
	g.6267G/A	-	1
6			
(rs6025601)	g.9348T/T	9	8
	g.9348T/C	1	2
9			
(rs6070122)	g.16392C/C	9	0
	g.16392C/G	1	0
	g.16382A/A	10	9
	g.16382A/G	0	1
11.2			
(rs62204256)	g.26874G/G	6	6
	g.26874G/A	4	4
	g.26945T/T	9	9
	g.26945T/C	1	1

studies deliver valuable data about methylation pattern establishment, alterations and gene expression regulations our study shows that the basis of methylation and its influence on the phenotype still remains unknown in Silver-Russell syndrome.

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