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Evidence against a major role of *PEG1/MEST* in Silver–Russell syndrome

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Silver–Russell syndrome (SRS) is a heterogeneous disorder characterised by interauterine and postnatal growth retardation, with or without additional dysmorphic features. Most cases are sporadic but a few familial cases have been described. A subset of patients exhibit maternal uniparental disomy for chromosome 7 (mUPD7) strongly suggesting that genomic imprinting plays a role in the aetiology of the disease. We and others have recently characterised the human *PEG1/MEST* gene, the first imprinted gene known to be located on chromosome 7. Although the function of *PEG1/MEST* is unknown, the paternal-specific expression of this gene and its location at 7q32, render it a promising candidate for SRS. As a prerequisite for mutation screening in 49 patients with SRS and 9 with primordial growth retardation (PGR), we determined the complete genomic structure of the *PEG1/MEST* gene which consists of 12 exons. Apart from one silent mutation and two novel polymorphisms, nucleotide changes were not detected in any of these patients. Moreover, methylation patterns of the 5' region of *PEG1/MEST* were found to be normal in 35 SRS and 9 PGR patients and different from the pattern seen in patients with mUPD7. These findings strongly argue against a role of *PEG1/MEST* in the majority of Silver–Russell syndrome cases.

Keywords: *PEG1/MEST*; Silver–Russell syndrome; uniparental disomy; imprinting

Introduction

Silver–Russell syndrome (SRS) is characterised by intrauterine as well as postnatal growth retardation.[1–3] Apart from PGR, most SRS patients have a triangular face, delayed bone maturation, hemihypertrophy and clinodactyly of the little fingers. Other less frequent anomalies are hypospadia, café-au-lait spots, syndactyly between the second and third toes, male hypoplasia and excessive sweating. None of these symptoms is obligatory, however; SRS patients may have PGR without any additional features. Most SRS cases are sporadic, but familial cases also exist. Both dominant and recessive inheritance have been reported.[4–6] The molecular basis of this disorder is unknown but its association with different chromosomal aberrations argues for

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genetic heterogeneity. In particular, two cases with severe SRS and balanced translocations disrupting the band 17q25,[7,8] one girl with SRS and a ring chromosome 15 involving deletion of the *IGF1R* gene[9] and one patient with Silver–Russell syndrome-like features and an interstitial deletion of 8q11-q13[10] have been found. Moreover, numerous studies have shown that maternal UPD7 is associated with SRS or PGR,[11–17] whereas paternal UPD7 does not give rise to growth retardation.[18]

Recently, we and others have isolated the imprinted human *PEG1/MEST* gene.[19,20] The gene has been localised to chromosome 7q32 and in all foetal tissues the expression was confined to the paternal allele.[19] The precise function of *PEG1/MEST* is unknown although the presence of motifs found in alpha/beta-hydrolase-fold enzymes points to a relationship with this family. Three strong lines of evidence have implicated *PEG1/MEST* as a candidate gene for Silver–Russell syndrome. Firstly, *PEG1/MEST* is the first imprinted gene discovered to date which is located on chromosome 7. Secondly, its expression is confined to the paternally derived allele and therefore inactive in SRS patients with mUPD7. Thirdly, targeted inactivation of the paternally derived mouse *Peg1/Mest* allele results in viable but growth retarded embryos (L Lefebvre and MA Surani, personal communication).

In this study, we have screened 49 patients with SRS and 9 with PGR, all with confirmed biparental inheritance of chromosome 7, for mutations and methylation changes in the *PEG1/MEST* gene in an attempt to substantiate its role in these disorders.

Materials and Methods

Patients

DNA was extracted from blood samples as described by Miller *et al*.[21] Maternal UPD7 had been excluded for all patients by simple repeat analysis (own results,[15].

Genomic Structure of PEG1/MEST

*Eco*RI fragments of cosmid ICRFc113K246Q4 that was known to contain the complete *PEG1/MEST* gene were ligated to pT7T318U (Pharmacia) and sequenced. One *Eco*RI fragment of 8.5 kb cloned in both orientations was further subcloned by religations of *Bam*HI, *Hind*III, *Pst*I, *Sma*I and *Xba*I digests. Sequencing was performed with standard vector primers using either the *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems) and analysed on ABI 377 automated sequencer, or the Thermo Sequenase cycle sequencing kit (Amersham) with IRD-41 labelled primers and analysed on the Li-Cor (MWG-Biotech) automated sequencer.

Single Strand Conformation Analysis

The primers for PCR analysis were designed to cover the putative promoter region, all exons as well as the intron-exon boundaries of *PEG1/MEST* as outlined in Table 1. For radioactive SSCP analysis the primers were endlabelled with T4 polynucleotide kinase (Gibco BRL) and [γ^{32}P]ATP in 10 $\times$ kinase buffer. PCR fragments were separated on 5% polyacrylamide-bisacrylamide (19:1) gels with and without 10% glycerol at 45 W, 4°C. Gels were transferred to filter paper, dried, and exposed to X-ray film for 10–24 h. Part of the exons were analysed by non-radioactive SSCP using the Multiphor II system (Pharmacia). 1–2 μl of PCR product was

Table 1 PCR primers for human *PEG1/MEST* exons

Exon	*Forward primer*	*Reverse primer*	*Size (bp)*	T_a	$MgCl_2$
*5′#	gggagcagcggggtcttgg	cagctgccgcagaggaggtg	270	65°C	1.5 mM
*1	aagtcggtgcccactcgctc	cgcagcgttgcagagcacc	229	61°C	1 mM
*1	gcgcacgccggagtggc	ccctagtcccagggccgc	239	58°C	3 mM
2	caatagtccacttcttacc	tgcttacagtgggtctctgc	204	56°C	2 mM
3	gattttaacatcttcgagg	ggccgagatcttttaatc	198	50°C	3 mM
4	gaaagggaggggcaggagc	ccctcatagttgcgtctgcc	188	59°C	2 mM
5	1.gaaagggaggggcaggagc	gatacaaacagtccagaaac	488	63°C	2 mM
5B	2.tgtcctcatgacttcatcc	gatacaaacagtccagaaac	280	58°C	2 mM
6	ggtttagtttgctcagctac	cccttctagattacacccac	151	55°C	2 mM
7	cagtgagatcccgtctc	ttacgtcagtacaaatcc	165	57°C	2 mM
8	ttcctctgggttcttgagc	acgacattgctctaggcc	156	52°C	3 mM
9	gttctcctcacacttatc	catcacaaaaacacttctc	192	50°C	3 mM
10	ctgtaacagtaaaggtttc	tactttaaccctatagtcc	282	57°C	3 mM
11	gtagaacagatgtgagagc	gtcaggatgagatactaac	263	50°C	3 mM
12	gctccagcctcaagttc	caatttgagtaaagtggac	292	50°C	3 mM

*Amplification of the 5' putative promoter region (#) and exon 1 was performed including 10% glycerol and 3.5% formamide. All primers are shown 5' to 3'. Exon 5 was amplified with two sets of forward primers, the first forward primer was identical to that of exon 4. The product from this reaction was used as template in the second PCR.

denatured in 95% formamide, 10 mM EDTA and loaded on an 8% polyacrylamide gel (29:1 or 49:1 acrylamide:bis-acrylamide) containing 2% glycerol. Electrophoresis was performed for 3–4 h at 250 V, 15°C or 20°C in 1 × TBE. For all exons resolution was optimised by applying both running conditions. Staining of the gels was performed according to standard technique.

Chromosome 7 Markers

Amplification of the dinucleotide repeats D7S2519 and D7S649 was performed with primer sets 5'-GGAGGTTAA-GATTTACAGACATG-3', 5'-TATCCTGTGTTTCCTTGTCCTG-3' and 5'-TGCTTTATTATGTCTGTTGTATG-3', 5'-ACTGAGT-CAAATTTGTAGAAGTT-3'. Thirty-five cycles of PCR consisted of denaturation at 94°C, 30 s, annealing at 56°C, 1 min, and elongation at 72°C, 1 min, in the presence of 1.5 mM $MgCl_2$ and [α^{32}P]dCTP. Products were size-fractionated at 60 W for 3 h on standard 6.6% denaturing polyacrylamide gels. Exposure was for 24–48 h.

Methylation Analysis

For methylation analysis the DNAs were digested with (*Hind*III + *Hpa*II) or (*Hind*III + *Msp*I) and transferred onto GeneScreen Plus membrane exactly as described previously.[19] The 4.3 kb *Hind*III probe containing the 5' portion of the *PEG1/MEST* gene was labelled by random priming in the presence of [α^{32}P]dCTP, and hybridisations were performed at 65°C. Washing was at hybridisation temperature in 2XSSC/0.1%SDS (2 × 10 min) followed by 1XSSC/0.1%SDS for 15 min. Autoradiograms (Kodak X-omat) were exposed for 16 h to one week.

Results

Genomic Structure of PEG1/MEST

As a prerequisite for mutation screening in the *PEG1/MEST* gene, we have first determined the complete exon-intron structure. Following subcloning of *Eco*RI fragments of the cosmid ICRFc113K246Q4, which had been isolated from a chromosome 7 specific library previously and had been shown to contain the entire gene,[19] *PEG1/MEST* positive fragments were sequenced. Alignment of the genomic and cDNA sequences revealed that the *PEG1/MEST* gene contains 12 exons and encompasses a genomic region of approximately 13 kb. Exon-intron junctions are given in Table 2 and the structure of *PEG1/MEST* is shown schematically in Figure 1.

Mutation Detection by Single Strand Conformation Polymorphism (SSCP)

SSCP analyses were performed to screen for mutations in the putative promoter region and all 12 exons of the *PEG1/MEST* gene. To include exon-intron junctions, primers were designed in the corresponding introns. In total, three bandshifts were detected, one in the putative promoter region and the others in exon 11 and 12, respectively. Sequencing of exon 11 and exon 12 fragments revealed a single nucleotide change T1065C, which is a silent mutation in exon 11 and a G1272C substitution which is located in the 3'UTR of the gene. The bandshift in the putative promoter region was present in the DNA of three patients and was also found in 15% of control DNAs. No other alterations were detected in the 58 patients tested.

In order to exclude the possibility of the patients being distantly related, we investigated two polymorphic CA-dinucleotide repeat markers, D7S2519 (accession No. Z54039[22]) and D7S649 (accession No. Z23771,[23]) which are located in the vicinity of the *PEG1/MEST* gene.[24] No evidence for allelic association

Table 2 Exon-intron organization of the *PEG1/MEST* gene

Exon number	*cDNA position*	*Exon size (bp)*	*Splice junctions*	
			3′ splice site	*5′ splice site*
1	1–244	244	CCAGCA	CCGCAG/**gt**gagt
2	245–399	155	ctac**ag**/GATGAG	ACCAAG/**gt**aaga
3	400–479	80	tcct**ag**/ACTCTG	TACAAG/**gt**aatg
4	480–557	78	ttgt**ag**/ATTTGG	AAACCG/**gt**aagc
5	558–694	137	cctc**ag**/AGACCT	CTACAG/**gt**cnnn
6	695–753	59	ttct**ag**/GTACAA	ATGGAG/**gt**aatt
7	754–794	41	ctac**ag**/GTATCT	CAAAAG/**gt**tggt
8	795–865	71	ctcc**ag**/CTACTC	TCGAGG/**gt**aagt
9	866–967	102	tttc**ag**/TCTCAC	TGACAG/**gt**aaga
10	968–1044	77	ctac**ag**/TCTCTT	TCCCCA/**gt**gagt
11	1045–1108	64	ttct**ag**/TTCATT	GTACAG/**gt**gagt
12	1109–1226	118	cact**ag**/GAAAAC	TTCTGA
3′UTR	1227–2460			

The cDNA positions refer to GenBank accession No. Y11534.

could be obtained which argues against a monophyletic origin of SRS.

Methylation Analysis in Silver–Russell Syndrome and Primordial Growth Retardation

To search for aberrant methylation patterns in the 5' CpG island of the *PEG1/MEST* gene resulting from 'imprinting mutations', we performed Southern blot hybridisations with DNA from SRS and PGR patients as target and a 4.3 kb *Hind*III fragment as probe. DNAs were digested with *Hind*III followed by digestion with either *Msp*I or its methylation-sensitive isoschizomer *Hpa*II. *PEG1/MEST* alleles derived from the mother give rise to a 4.3 kb fragment or to fragments of 4.3, 2.3 and 2 kb due to partial methylation of one *Msp*I site in the vicinity of the CpG island.[19] Paternally-derived alleles are completely unmethylated and therefore digested by *Hpa*II which is indicated by the presence of a 2.3 kb fragment and several smaller fragments. As shown in Figure 2, all patients showed a methylation pattern identical to that of normal control DNA and different from the pattern observed in patients with mUPD7. This indicates that the paternal allele is not deleted and that both *PEG1/MEST* alleles exhibit normal parent-of-origin specific methylation patterns.

Discussion

Recently we have shown that the human *PEG1/MEST* gene is imprinted and expressed from the paternally derived allele. The gene has been mapped to chromosome 7q32.[19]

The findings of Kotzot *et al*,[15] Preece *et al*[16] and Eggermann *et al*,[17] demonstrating that a subset of patients with SRS and primordial growth retardation carry two maternal chromosomes 7 while lacking the paternal counterpart, and the previously described association between mUPD7 and short stature, suggest that an imprinted gene on chromosome 7 is involved in this disorder. *PEG1/MEST* expression from the paternally-derived allele and its location on chromosome 7q, as well as the finding that in the mouse paternal inheritance of the inactivated *Peg1/Mest* allele leads to foetal growth retardation, renders it an excellent candidate gene for SRS.

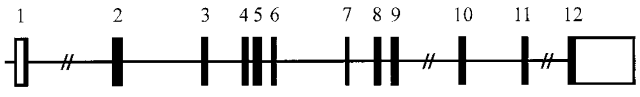

Figure 1 *Genomic organization of the human PEG1/MEST gene. Exons are indicated by filled boxes, the 5' and 3' untranslated regions are depicted as open boxes.*

In order to find mutations in the *PEG1/MEST* gene, we first determined the genomic structure which was found to consist of 12 exons. Mutation analysis by SSCP and subsequent sequencing revealed two novel polymorphisms and a single nucleotide change resulting in a silent mutation in exon 11. Very likely, none of these sequence variants is of functional importance and no other mutations were found. A monophyletic origin of the SRS patients is most unlikely since no evidence for allelic association could be obtained by analysing two highly polymorphic microsatellites which are located in the vicinity of *PEG1/MEST*.

Silver–Russell syndrome patients with mUPD7 lack the paternally expressed *PEG1/MEST* gene product. Silencing of the maternally derived *PEG1/MEST* allele is reflected by maternal-specific methylation of the CpG island at the 5' portion of the gene. During gametogenesis, parental-specific (methylation) marks have to be reset according to the gender of the individual. In a minority of cases this resetting fails, resulting in an incorrect parental methylation profile. Such so-called 'imprinting mutations' have been identified in patients with imprinted gene disorders such as Prader-Willi syndrome (PWS), Angelman syndrome (AS) and Beckwith-Wiedemann syndrome.[25, 26] In PWS and AS the imprinting mutations arise in the imprinting center which regulates imprinted gene expression and methylation over a distance of several hundred kilobases.[27] In this region, tissue-specific imprinting for *UBE3A* has been observed with expression from the maternally-derived allele in the brain, but biallelic expression in other foetal tissues and adult lymphocytes and fibroblasts.[28, 29] The *PEG1/MEST* gene is also biallelically expressed in adult blood cells but parent-specific methylation differences are still present. In all SRS patients tested, analysis of the critical 5' region of the *PEG1/MEST* gene revealed methylation patterns identical to that of normal control DNAs. These findings rule out silencing of *PEG1/MEST* in the SRS by aberrant methylation of the putative promoter region caused by 'imprinting mutations'. Taken together, the absence of mutations in the *PEG1/MEST* putative promoter region and the entire coding region including exon/intron boundaries and the normal methylation patterns of the 5' CpG island, strongly argue against an involvement of the *PEG1/MEST* gene in the majority of SRS cases. We cannot, however,

completely rule out a role for *PEG1/MEST* in SRS. Patients with mUPD7 lack the *PEG1/MEST* gene

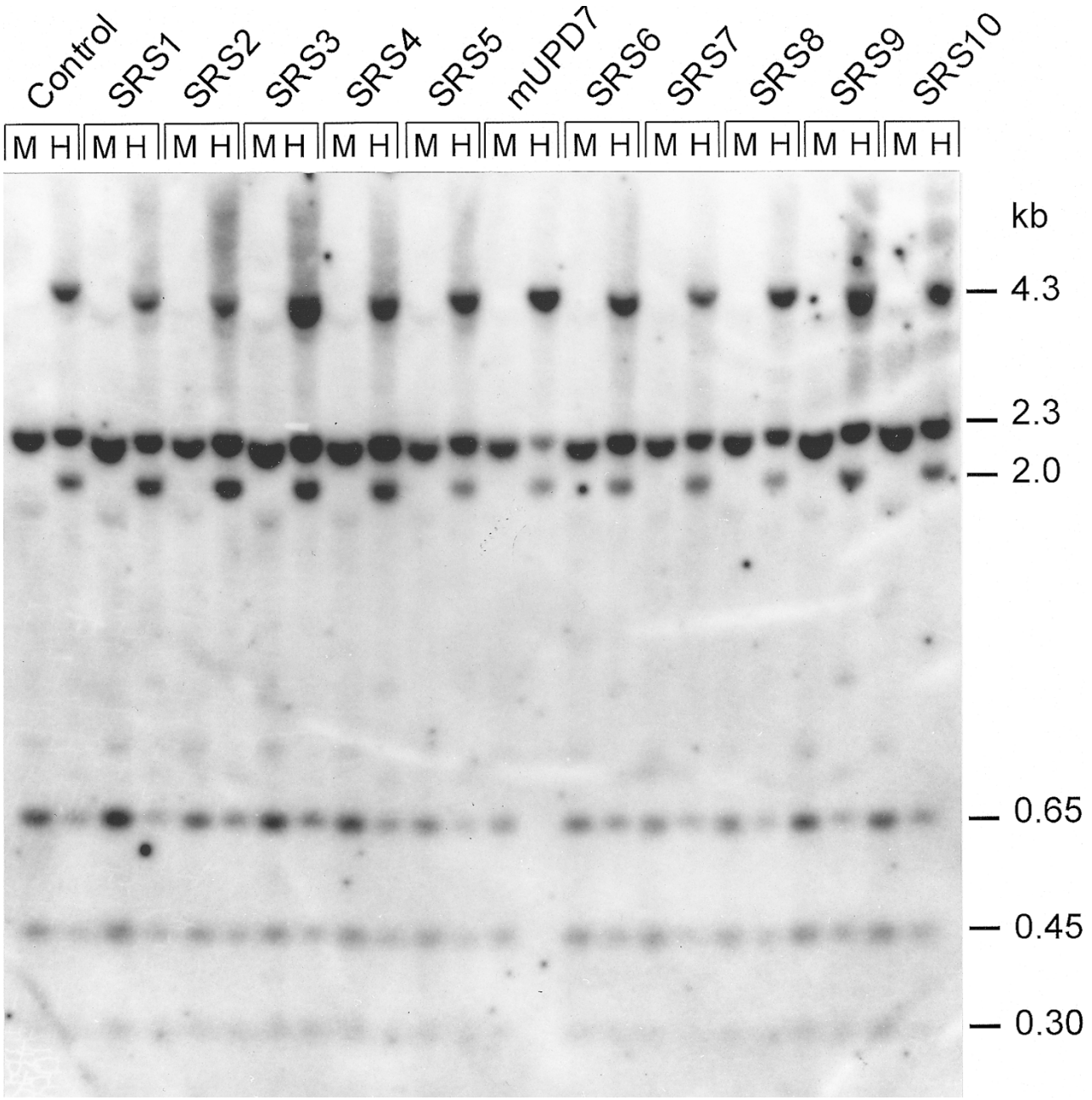

Figure 2 *Methylation analysis of the 5' region of human PEG1/MEST in sporadic SRS patients. Southern blot containing genomic DNA of sporadic SRS patients, a normal control and a patient with mUPD7 digested with either (*Hind*III* + Msp*I) or (*Hind*III* + Hpa*II) were hybridized with a 4.3 kb* Hind*III fragment derived from the 5' end of the PEG1/MEST gene encompassing the maternal-specific methylated CpG island. Digestion with* Msp*I reduces the 4.3 kb* Hind*III band to fragments of 2.3, 0.65, 0.45 and 0.3 kb. Because of their small size a few fragments could not be detected. Upon digestion with* Hpa*II the unmethylated paternal allele is completely digested giving rise to fragments identical in size to the* Msp*I derived fragments, whereas the methylated maternal allele remains either completely undigested or is digested on a partially methylated* Msp*I site located outside the CpG island giving rise to a 2.3 and a 2.0 kb band. All SRS patients show a pattern identical to that of a normal control with both a maternal and a paternal methylation profile. In contrast, a mUPD7 patient shows, after digestion with* Hpa*II, only a maternal methylation pattern with absence of the smaller paternally derived bands.*

product and disruption of the paternal allele is suspected to have a negative effect on growth as deduced from the gene targetting experiments in mice. Since SRS is heterogeneous, mutations in any of the genes could be present in a relatively small number of SRS cases.

In mice, maternal duplication of the proximal region of chromosome 6, including *Peg1/Mest* leads to embryonic lethality, whereas paternal duplication of this region is viable.[30] It is not yet understood why mice with maternal disomy for this region die in embryogenesis whereas in humans mUPD7 is associated with a relatively mild phenotype. The leaky expression of the maternal *PEG1/MEST* gene observed in humans but not in the mouse could be sufficient to avoid embryonic lethality.[20,31] In mice, imprinting and X-inactivation processes are, in general, more strictly regulated than in humans which might explain the more severe problems observed on parent-specific duplications of imprinted regions.

Imprinted genes tend to be clustered, as documented for the human and mouse genome. In humans, two major clusters, one on chromosome 15q11-q13 and the other on chromosome 11p15.5, have been identified. It is therefore tempting to speculate that apart from *PEG1/MEST*, the relevant region on chromosome 7 harbours additional imprinted genes, one of which may be involved in SRS and/or primordial growth retardation. The imprinting status of expressed sequences from this region is currently being investigated.

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Anne M Riesewijk and Nadya Blagitko contributed equally to the work reported in this paper.

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