Electronic letter

J Med Genet 2000;**37** (http://jmedgenet.com/cgi/content/full/37/12/*e*44)

Exclusion of a disease relevant role of *PAX4* in the aetiology of Silver-Russell syndrome: screening for mutations and determination of imprinting status

EDITOR—Silver-Russell syndrome (SRS) describes a uniform malformation syndrome characterised by intrauterine and postnatal growth retardation (IUGR/PGR), asymmetry of the head and limbs, a small triangular face, and other less constant features. The majority of the 400 cases described so far occurred sporadically, but some familial cases indicate a genetic cause of the disease. In rare cases, chromosomal aberrations have been found. Although no uniform pattern is apparent,[1] five cases have recently been reported involving chromosome 7.[2–6] A subset of 7-10% of SRS patients shows maternal uniparental disomy (mUPD) for the whole of chromosome 7,[7] thus indicating the involvement of at least one imprinted gene on this chromosome. Mutations in this gene or imprinting mutations may contribute to the SRS phenotype. So far, two imprinted genes localised in 7q32 (*MEST*/*PEG1*, *COP-G2*) have been excluded as candidate genes for SRS.[8 9]

PAX4 is a member of a highly conserved gene family,[10] and has been mapped to 7q32.[11] Evidence of the crucial role of *PAX* genes in organogenesis and in differentiation is provided by mouse developmental mutants[12] as well as by human diseases (*PAX2*, kidney abnormalities, optic nerve coloboma; *PAX3*, Waardenburg syndrome types I and III; *PAX5*, small lymphotic lymphomas; *PAX6*, aniridia; *PAX9*, optic nerve coloboma).[10 13 14]

A role for *PAX4* in the aetiology of diabetes type II has recently been dismissed by investigations in a group of French patients as well as in a population of 116 unrelated Ashkenazi Jews affected by the disease.[15 16] Furthermore, *PAX4* has been ruled out as a possible candidate gene for the Wolcott-Rallison syndrome.[17]

The *PAX4* gene product contains two DNA binding motifs, a paired domain which consists of three alpha helices, and a paired type homeodomain with a helix turn helix motif; owing to its localisation in the nucleus and its ability to bind DNA, it might serve as a transcription factor.[10]

PAX4 is expressed predominantly in placenta and skeletal muscle; fewer transcripts have been observed in the heart.[15] Detection of *Pax4* transcripts in the early pancreas of mice in combination with results of knock out experiments indicate an important role for *Pax4* during development: mice homozygously deficient for *Pax4* suffer from growth retardation and dehydration at birth and die within three days.[18] Its expression in pancreatic island progenitor cells, which are the common progenitor for beta and delta cells, controls the initial processes of endocrine differentiation. In the mature endocrine pancreas, it might contribute to the maintenance of the differentiated state of beta cells, since then *Pax4* is restricted to this cell population.[18]

So far, there is no information available about the imprinting status of *PAX4*. Since imprinted genes tend to be clustered,[9] the close proximity of *PAX4* to *MEST*/*PEG1* and *COP-G2*, which have recently been shown to be paternally expressed, renders its imprinting possible. Taking into account its function, the very restricted spatial and temporary pattern of expression during embryogenesis, and its localisation in 7q32, *PAX4* was considered to be a good candidate gene for SRS.

Our study population consisted of 50 SRS patients in which diagnosis was ascertained according to Wollmann *et al*.[1] Cytogenetic analyses by G banding on peripheral blood lymphocyte cultures of these patients did not show any abnormalities. A maximum of 53 unrelated, healthy probands of German origin were investigated as controls. Genomic DNA from patients and controls was isolated

Table 1 Data on the PAX4 fragments analysed in this study and PCR conditions

Exon size (bp)	*Primer (5'>3')*	*Cosmid AC000359 (exon positions)*	*mRNA AF043978*	*Ta (°C)*	*$MgCl_2$ (mM)*
5'UTR	atacctctgtctcacgccca ggcttcctgtccccatcactg	31609-31362	1–206	63.5	1,5
1 (120)	atacctctgtctcacgccca ggcttcctgtccccatcactg	31074-30881 (31042-30923)	207–326	63.5	1,5
2 (216)	catgcctcacctctccctg tcttttccagccccagtgtg	30653-30363 (30617-30402)	327–542	63.5	1,5
3 (76)	atctctccactcaacaccct acgagaaagggctttgagaa	30110-29927 (30079-30004)	543–618	58	1,5
4 (126)	atctgaccagaggaatcacc tgtcacactgaggactctct	29460-29231 (29043-29278)	619–744	61	1,5
5 (83)	ttggggttgtagcaggtggg cttcctccttgtggtttgta	29148-28929 (29053-28971)	745–827	60	1,5
6 (70)	gatgtcaggcccaaggaagggtcaa gatgactgagcggcagatggatg	28730-28501 (28612-28543)	828–897	67	1,5
7 (56)	caaccttcctgcctctcacc cccatgagcccttcagtctt	27610-27420 (27529-27474)	898–953	65	1,5
8 (142)	ctggaagctaaggttcttct tcagtcgaccctccctacat	27243-27011 (27205-27064)	954–1095	61	1,5
9 (143)	aagctctctctatttgtccc cacacaggagggagcatca	27660-26407 (26735-26593)	1096–1238	65	1,5
3'UTR1	cggcccactactgtatggct agagtgggcataggggtgctcata	26760-26407	1239–2020	59	1,5
3'UTR2	tcgatgcctctctggcgcat aatgccatgatataatatca	26451-26050		55	3
3'UTR3	tgtatgtatgtgtgcatgcg cccctggagcatgtataaat	26122-25773		53	3

Ta, annealing temperature; $MgCl_2$, final concentration.

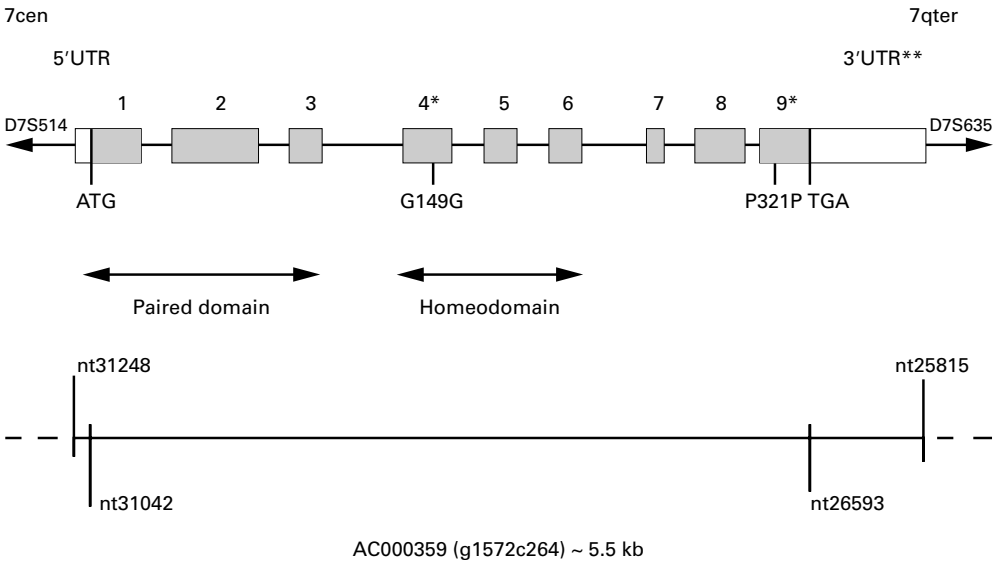

Figure 1 Schematic representation of the PAX4 gene[15 17] and the newly detected polymorphisms.

from peripheral blood lymphocytes. RNA was isolated from five SRS patients using the QiaRNeasy Blood Kit.

Information about the genomic structure of the *PAX4* gene was obtained by performing a BLAST search in public databases with mRNA g5453849[17] as a probe. The identified PAC clone g1572c264 (GenBank Accession No AC000359) was used to choose PCR primers located in introns. Updating of the exon/intron structure and coding/non-coding regions was performed according to the more detailed information published by Tao *et al.*[15]

For cDNA synthesis, RNA probes (1 µg) were treated with DNase I, subsequently denatured at 94°C for five minutes, and immediately chilled on ice. Reverse transcription was carried out using random hexanucleotide primers (1.5 µg), 1 × first strand buffer, 1 vol dithiothreitol, 10 mmol/l each dNTP, 200 U Superscript reverse transcriptase (Gibco BRL), and 80 U RNase inhibitor (Gibco BRL). These were added to a final volume of 38 µl, incubated at 42°C for one hour, and the reaction terminated by heating for 15 minutes. To detect possible DNA contamination of the RNA samples, we subjected half of each probe to the reaction without the addition of reverse transcriptase.

To produce overlapping PCR fragments of the coding region, 13 sets of primers were chosen encompassing the whole coding region consisting of 9 exons, the exon/intron boundaries, as well as the 5' and 3' untranslated regions. Fragment sizes were between 200 and 440 bp.

In case of detected variants, up to 50 SRS patients were investigated for each fragment as well as a maximum of 53 controls. Standard PCR was carried out in a total volume of 25 µl containing 80 ng of genomic DNA, 20 pmol of each primer, 50 mmol/l KCl, 10 mmol/l Tris-HCl (pH 8.3), 1.5-3 mmol/l $MgCl_2$, 0.01% gelatine, 200 µmol/l of each dNTP, and 1 U *Taq* Polymerase (Gibco). Primer sequences and PCR conditions are listed in table 1.

Mutation screening was performed by single strand conformation polymorphism analysis (SSCA): 3 µl of PCR products were mixed with 7 µl of denaturing solution containing 80% deionised formamide, 0.0125% bromophenol blue, and 0.75% Ficoll 400, and denatured for five minutes at 94°C. Samples were subsequently chilled on ice and then loaded on 10% (49:1) polyacrylamide gels with and without glycerol (5%). They were allowed to run for 16-18 hours at 7 V/cm, both at 4°C and room temperature. Bands were visualised by silver staining.

For sequencing, symmetrical PCR products were purified by ultrafiltration using the Qiaquick PCR purification kit and concentrated from 100 to 40 µl. One of the two strands was then selectively amplified from 70 ng of the purified products using the BigDyeTerminator kit (Perkin Elmer).

Excluding null alleles, the alleles of polymorphic loci show codominance, providing complete genotypic information from which estimates of Hardy-Weinberg deviation and genetic structuring of groups can readily be calculated. The statistical significance of Hardy-Weinberg deviation, intergroup differences of allele, and genotype frequencies were determined using exact test procedures in Raymond and Route's GENEPOP package.[19 20]

We screened the nine exons and the 3'UTR of *PAX4* for disease related mutations and variants useful for expression studies (fig 1, table 1).

Table 2 Detection and characterisation of new DNA sequence variants in the human PAX4 gene

		Allele frequency (chromosomes)	
Fragment	*Nucleotide position**	*Controls*	*SRS patients*
Exon 4	nt29366G>A (G149G)	0/56	1/88
Exon 9	nt26663G>T (P321P)	15/106	18/88
3'UTR	nt26382C>T	10/66	28/100
	nt26112A>G	11/14	43/66

*Nucleotide positions based on cosmid g1572c264 (AC000359).

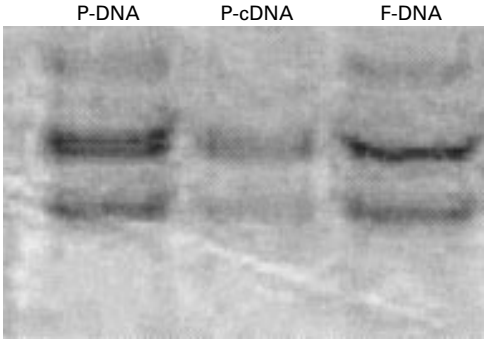

Figure 2 SSCA of polymorphism nt26382C>T in 3'UTR1: DNA and cDNA of a heterozygous SRS patient show the same band pattern; the father homozygous for the wild type allele shows a different pattern (P, SRS patient; F, father).

Using SSCA, we identified four polymorphisms distributed in the coding region and the 3'UTR (table 2, fig 2); however, we may have missed some variants relying on SSCA as the only screening method since its sensitivity is less than 100%. The variant in exon 4 (nt29366G>A) was very rare, thus emphasising the strong conservation of this gene, while the variation in exon 9 (nt26663G>T) occurred frequently as well as the two polymorphisms in the 3'UTR (nt26382C>T, nt26112A>G). Both base pair substitutions in the coding region were identified as silent mutations (G149G, P321P).

The patterns of allele and genotype frequencies were highly consistent for all loci and for both the patient and control group. The observed genotype distributions showed no significance compared with Hardy-Weinberg expectation in the patient or in the control group. Furthermore, the allele and genotype frequencies did not differ significantly between the patient and control group for all loci ($p>0.06$).

To determine the imprinting status of *PAX4* and to distinguish between mono- and biparentally expressed alleles, the identified variants were investigated at the RNA level: Two SRS patients heterozygous for polymorphisms nt26382C>T and nt26663G>T, respectively, showed no reduction to homozygosity at the cDNA level; thus, biallelic expression of *PAX4* in peripheral blood lymphocytes could be shown. Additional proof came from a SRS patient with mUPD7, since several fragments of the *PAX4* transcript could be amplified from RNA extracted from peripheral blood lymphocytes, thus excluding the possibility of maternal imprinting of this gene. So far, these results allow no conclusions about any tissue or time restricted pattern of genomic imprinting for *PAX4*, and further investigations will have to follow.

To sum up, we exclude *PAX4* as a major candidate gene for SRS, since we could not detect any disease relevant mutations in our study population. Nevertheless, there remains the possibility that we missed some rare mutations, which might play a relevant role in a small number of SRS patients, since SRS is likely to originate from a heterogeneous background.

In addition to *MEST*/*PEG1* and *COP-G2*, *PAX4* is a further gene in 7q32 that has been excluded as a candidate gene for SRS. Recent publications describe duplications of 7p12-p14 in SRS patients, indicating that putative SRS genes are located in this region, which might be an important argument to focus further investigations on the short arm of chromosome 7.

This work was supported by the START research programme of the Technical University of Aachen and a grant from Pharmacia & Upjohn, Sweden. The authors wish to thank the "Bundesverband Kleinwüchsiger Menschen" and the SRS families who participated in this study.

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