

Mutation Analysis of the RSK2 Gene in Coffin-Lowry Patients: Extensive Allelic Heterogeneity and a High Rate of De Novo Mutations

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Summary

Coffin-Lowry syndrome (CLS) is an X-linked disorder characterized by severe psychomotor retardation, facial and digital dysmorphisms, and progressive skeletal deformations. By using a positional cloning approach, we have recently shown that mutations in the gene coding for the RSK2 serine-threonine protein kinase are responsible for this syndrome. To facilitate mutational analysis, we have now determined the genomic structure of the human RSK2 gene. The open reading frame of the RSK2 coding region is split into 22 exons. Primers were designed for PCR amplification of single exons from genomic DNA and subsequent single-strand conformation polymorphism analysis. We screened 37 patients with clinical features suggestive of CLS. Twenty-five nucleotide changes predicted to be disease-causing mutations were identified, including eight splice-site alterations, seven nonsense mutations, five frameshift mutations, and five missense mutations. Twenty-three of them were novel mutations. Coupled with previously reported mutations, these findings bring the total of different RSK2 mutations to 34. These are distributed throughout the RSK2 gene, with no clustering, and all but two, which have been found in two independent patients, are unique. A very high (68%) rate of de novo mutations was observed. It is noteworthy also that three mutations were found in female probands, with no affected male relatives, ascertained through learning disability and mild but suggestive facial and digital dysmorphisms. No obvious correlation was observed between the position or type of the RSK2 mutations and the severity or particular clinical features of CLS.

Introduction

Coffin-Lowry syndrome (CLS) (MIM 303600) is a rare syndromic form of X-linked mental retardation, with some diagnostic features, such as skeletal abnormalities. Typically, male patients are of short stature and exhibit a characteristic coarse face with a prominent forehead, orbital hypertelorism, epicanthic folds, thick lips, a thick nose septum with anteverted nares, and irregular or missing teeth. Their large and soft hands with lax skin and tapering fingers are usually diagnostic features. The most frequent skeletal changes are delayed bone development, spinal kyphosis and/or scoliosis, and pectus carinatum or excavatum. There is marked variability in the clinical expression of the disease (Coffin et al. 1966; Lowry et al. 1971; Temtamy et al. 1975; Fryns et al. 1977; Hunter et al. 1982; Gilgenkrantz et al. 1988; Krajewska et al. 1988; Young 1988; Hartsfield et al. 1993).

By using a positional cloning approach, we recently identified the gene mutated in Xp22.2 in CLS patients (Trivier et al. 1996). It encodes RSK2, a growth factor-regulated serine-threonine protein kinase, acting in the ras-mitogen-activated protein kinase signaling pathway (Blenis 1993). The human RSK2 cDNA encodes a protein of 740 amino acids containing two nonidentical kinase catalytic domains (Jones et al. 1988; Bjorbaek et al. 1995).

Initial screening of the RSK2 gene in CLS patients resulted in the identification of 11 different mutations, 8 of which were predicted to result in protein truncation; the remaining 3 resulted in amino acid substitutions, affecting highly conserved residues within the N-terminal ATP-binding site and within two known phosphorylation sites. All these mutations were predicted to have detrimental effects on the transduction function of the RSK2 protein, suggesting that loss of function of RSK2 is responsible for the disorder (Trivier et al. 1996).

To gain more insight into the mutational spectrum in CLS and to see whether some genotype/phenotype correlation emerges regarding either severity of the disease or expression of uncommon clinical features, we have determined the intron-exon structure of the human RSK2 gene and have extended mutation screening to a

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Table 1

Clinical Data

	STATUS OF PATIENT*																													
	BL4	BC72	DZ3	DX36	DX56	DX84	DZ12	BC55	DZ34	DZ15	DZ28	DZ20	DZ47	DZ68	CU94	DZ50	DZ86	DZ21	DZ37	DZ23	DX15	CV59	DX29	DX43	DX96	DZ6	BQ20	CE21	DX68	
Sex	M	M	M	M	M	F	M	M	M	M	M	M	M	F	M	M	M	M	M	F	M	M	M	M	M	M	M	M	M	M
Age at examination (years)	22	14	4	10	33	12	8	22	17	9	21	5	12	38	8	4	7	28	20	8	15	8	21	16	11	3	8	25	24	
Neurological effects:																														
Mental retardation	S	S	S	Mo	S	LD	S	S	S	S	Mo	Mi	Mo	Mi	Mo	S	Mi	Mi	S	LD	Mo	Mi	Mo	Mo	S	S	S	Mo	S	
Developmental delay	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Speech delay	+	+	+	0	+	NA	+	+	+	+	+	NA	+	NA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Hypotonia	+	+	+	+	+	NA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0	+	+	+	NA	+	+	
Ventricular dilatation	+	NA	0	0	NA	NA	NA	NA	+	0	NA	NA	NA	NA	NA	+	NA	NA	+	NA	NA	NA	+	NA	NA	+	NA	0	0	
Sensorineural hearing deficit	NA	NA	NA	0	+	0	NA	0	+	NA	0	+	0	0	0	NA	0	0	0	0	0	+	0	0	0	+	0	+	0	
Seizure	+	+	0	0	0	0	NA	0	0	NA	+	NA	0	0	NA	NA	0	0	0	+	+	0	+	0	+	0	+	+	0	
Cataplexia	NA	NA	0	0	0	NA	NA	NA	0	NA	+	NA	+	+	NA	NA	0	0	0	NA	0	0	NA	NA	+	0	NA	+	0	
Growth:																														
Small stature	+	+	+	NA	NA	+	+	+	+	+	+	+	NA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0	+	+
Stooped posture	+	NA	NA	+		0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0	NA	0	+	+	NA	0	+	NA	0	+	0	
Facial features:																														
Microcephaly	+	0	+	+	0	+	+	+	0	0	NA	NA	0	NA	+	+	0	+	0	0	NA	+	+	0	+	0	0	NA	0	
Broad nose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Anteverted nares	+	+	+	+	+	+	+	+	+	+	+	NA	NA	+	NA	+	+	+	+	NA	+	+	+	+	+	+	+	+	+	
Large ears	0	NA	+	+	+	+	0	+	+	+	0	NA	+	+	+	NA	0	+	NA	+	NA	0	+	+	NA	+	NA	NA	+	
Hypertelorism	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0	+	+	+	+	+	
Down-slanted palpebral fissures	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	NA	0	+	+	+	+	+	+	+	+	+	+	+	+	
Thick/everted lips	NA	NA	+	0	+	+	+	+	+	+	+	NA	+	+	+	+	+	NA	+	+	+	+	+	+	+	+	+	+	+	
Large mouth	+	+	+	+	+	+	NA	NA	0	+	NA	NA	+	+	+	+	0	0	+	+	+	0	0	+	+	+	NA	+	+	
Frontal bossing	+	+	+	+	+	0	+	+	0	+	+	NA	+	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+	
Maxillary hypoplasia	+	0	+	+	+	0	NA	NA	NA	+	NA	NA	NA	+	NA	+	0	+	0	+	0	+	+	NA	0	NA	+	+	0	
High-arched/narrow palate	NA	+	NA	NA	NA	0	NA	NA	+	NA	+	NA	+	NA	+	+	+	NA	+	+	NA	NA	+	NA	NA	+	NA	+	NA	

Table 2
Intron-Exon Structure of the RSK2 Gene

EXON	POSITION ^a	SIZE	SPlice Junctions IN	
			3' Splice Site	5' Splice Site
1	?-70	?	...	GCTGAGgtgagtcagcagggcggc
2	71-126	57	cttgctgtgcttttcacagAATGGA	CAAACtgaagttaaaattttat
2	127-243	117	cttactgtttatctcaacagGAAGAA	GGAAAgtgaagtcagtgctgtgctc
4	244-325	82	ttctgtcatttcaatatagGTTTTT	TGAAAgtgaagtcagtcgctctattt
5	326-406	81	tacatacaatcttttatagTTCGAG	ATTATgtgaagtcagtcgctctcc
6	407-486	80	ttttcatttcttcattcagCTTTTC	AAAGAGgtatataattaatctctat
7	487-593	107	aattattgttttatttttagGTGATG	AGAAAgtgaagtcagtcgctctacta
8	594-631	38	ttttctgtttttttcagTATACT	TAACAGgtaaaaaaaaatcctcgt
9	632-774	143	atgatattgttttggcagATTTTCG	TTAATgtgaagtttgaatttggga
10	775-845	71	ttatttgccttctgtagTTTGAA	TCTTAAgtgaagtcaccttaaat
11	846-934	89	atgtggatttattatttagAGCCAA	GATTAGgtaaaaaaaaatcctcgt
12	935-999	65	gtgaatccattttttagtagGTGCAG	TGGAATgtgaagtcagtcgctctaac
13	1000-1102	103	cttttttttccccccagAAACTG	CCAAAgtgaagtcagtcgctctaac
14	1103-1227	125	cacttcttactttttcagATTCAC	GTTTCAGgtgagtcagtcgctctaac
15	1228-1353	126	aatttataatttttttagCAGTTA	GTGAAGgtgaagtcagtcgctctaac
16	1354-1443	90	ataaacatttgccttttagATTATT	AAGGATgtgaagtcagtcgctctaac
17	1444-1602	159	ttgatgttgaatttatagGTATAT	CAAGGGgtgaagtcagtcgctctaac
18	1603-1765	163	taatttgtattcaatttagGTGGTT	CCAGAGgtgaagtcagtcgctctaac
19	1766-1841	76	aatttgcacttttctagGTTTTA	TACCGGgtgagtcagtcagtcgctctaac
20	1842-1959	118	taattttattataaatttagTTACAC	GCAAAGgtgaagtcagtcagtcgctctaac
21	1960-2101	141	tttattattcccattcagGACCTG	GTAAAGgtgagtcagtcagtcgctctaac
22	2102-?	?	ctttatttctttgttcagGGTGCC	...

^a According to the numbering used by Bjorbaek et al. (1995).

large number of CLS patients. The coding region of the gene is divided into 22 exons. Genomic DNA from 37 unrelated patients, including 7 familial and 28 sporadic cases (for 2 patients the status was unclear), presenting highly suggestive features of CLS were analyzed for the presence of mutations. Twenty-one exons, carrying 97% of the RSK2 coding region, were explored by SSCP analysis and direct sequencing of the abnormal conformers. Twenty-five different mutations were identified. As expected for a severe X-linked disease, we found a high heterogeneity of mutations, widespread throughout the coding sequence. A very high proportion appear to be new mutations, and ~80% are predicted to result in truncated proteins. Evaluation of the available clinical data from the CLS patients gives no clear indication of correlation between genotype and phenotype.

Patients and Methods

Patients

Genomic DNAs of 37 unrelated patients with the diagnosis or suspected presence of CLS were screened for a mutation. They were referred, mainly consecutively, to the publication of the CLS gene identification (Trivier et al. 1996), by 35 different European, American, and Australian pediatricians or clinical geneticists. For these 37 patients, clinical information suggestive of CLS was available, and/or a mutation was found (table 1). It is

worth noting that 23 additional patients, negative for a mutation, were not included in the present report, since no clinical data and no photographs have been available on them; if clinical records can be obtained, they will be further studied. Most (28/35; for 2 the status was uncertain) of the 37 patients included in the present study were sporadic cases, including the 4 females (apart from the proband, there was no other affected male or obvious expressing female carrier).

Intron-Exon Structure of the RSK2 Gene

DNA from two YACs (2527D1 and 4BH4) was digested in two separate reaction mixtures, one with *EcoRI* and one with *XbaI*, and the resulting fragments were subcloned in a λ ZAP vector (Stratagene) digested with either *EcoRI* or *XbaI*. Ligation of the YAC fragments to the λ vector arms, packaging using the Gigapack III XL packaging extract (Stratagene), and infection of *Escherichia coli* XL1 blue strain were done according to the supplier's recommendations. Subclones were screened by hybridization with several different cDNA-derived fragments, which together covered the entire open reading frame. Positives clones were sequenced with exon-derived primers.

SSCP Analysis of CLS Patients

Primer sets for each exon are listed in table 2. PCR reactions were performed in 20- μ l volumes containing

100 ng of genomic DNA template; 1 × PCR buffer (Perkin-Elmer); 200 μM each dATP, dGTP, dCTP, and dTTP; 10 pmol of each primer; 0.3 mCi of dCTP α³²P; and 0.25 U of *Taq* DNA polymerase (Perkin-Elmer). PCR reactions were performed in a PTC 100 thermal cycler (MJ Research) and consisted of 30 cycles of 94°C for 10 s, the appropriate annealing temperature for each primer pair (table 1) for 10 s, and 72°C for 10 s. A final extension reaction was performed at 72°C for 5 min. PCR products were diluted 1/5 in formamide buffer (95% formamide/10 mM EDTA pH 8.0, 0.05% bromophenol blue, and 0.05% xylene cyanol), were denatured at 90°C for 5 min, and then were cooled rapidly. For each sample, 4 μl was loaded onto a 10% glycerol/6% polyacrylamide gel. The gels were run at 5 W for 10–12 h at 4°C. The PCR-amplified cDNA from patients with altered migration was sequenced directly on an ABI DNA sequencer. All sequences were determined on both strands.

Results

Intron-Exon Structure of the RSK2 Gene

Partial sequencing of subclones derived from two YACs, yWXD2527 and 4BH4, containing the human RSK2 gene, and comparison with the RSK2 cDNA sequence allowed us to identify 21 exon-intron boundaries interrupting the RSK2 coding region. The nucleotide and deduced amino acid sequences of all exons were identical to the cDNA sequence that had been reported elsewhere (Bjorbeak et al. 1995). The sequences of the exon-intron boundaries, as well as exon sizes, are given in table 2. All exon-intron boundaries conformed to the GT/AG consensus for intronic donor and acceptor splice sites. As reagents for mutational analysis, primers were designed, from the intronic sequences, to amplify each exon from genomic DNA. For amplification of the coding sequences contained within the first exon and the last exon, in each case one primer in the 5' UTR (flanking the ATG initiation codon) and one primer in the 3' UTR region (flanking the TAG stop codon), respectively, was chosen. For all but exon 1, they produced a single product of the predicted size; despite repeated trials with different primers, no unique fragment could be obtained for exon 1. Since chimerism of the genomic clones in the region immediately upstream of the translation-initiation codon could not be excluded, a search for cDNA clones extending into the 5' UTR was initiated. No human expressed sequence tag containing 5' UTR could be identified by database searches. However, screening of several human cDNA libraries by a PCR-amplified probe corresponding to the extreme 5' end of the coding region resulted in the identification of one clone that extended 50 nucleotides upstream of the translation-initiation co-

don, in the 5' direction. No differences were detected by comparison of this additional cDNA sequence versus the corresponding genomic sequence determined on the basis of the YAC subclone, excluding the possibility that failure to amplify exon 1 is due to chimerism. This sequence was revealed to be highly GC rich, with 84% guanines and cytosines. The primer sets used to amplify exons 2–22 of RSK2 from genomic DNA templates are listed, with their product sizes, in table 3.

RSK2 Mutation Analysis in CLS Patients

Exons 2–22 were amplified from the DNA of one affected individual from each of 37 families and were screened for mutations by means of SSCP analysis. Abnormal SSCP bands were found in 31 patients. Direct sequencing of both strands of the corresponding PCR products revealed, in 25 patients, sequence changes predicted to be disease-causing mutations. Table 4 summarizes the mutations observed in this study. In 15 patients (including 9 with a mutation), polymorphisms were identified.

Eight single-base substitutions were found at consensus splice sites, in seven different introns, in eight unrelated patients (table 3). Six occur at splice-acceptor sites, and two occur at donor sites. Two affected the same splice-acceptor site 5' of exon 9, one at position -2 (A→G) and the second at position -1 (G→T). All of these mutations were unique to the present study, and only one, the 326 -1G→C mutation present in a manifesting female proband (CN49) (without any affected male in her family), has been documented elsewhere, in one male patient (BB89; Merienne et al. 1998). The exon organization of the RSK2 coding sequence predicts that all but one (i.e., 326 -1G→C) of the splice-site mutations will result in the alteration of the reading frame, leading to the introduction of a premature termination codon. The 326 -1G→C mutation had previously been shown, in patient BB89, to lead to in-phase skipping of exon 5. Patients BB89 and CN49, sharing the 326 -1G→C mutation, had entirely different haplotypes around RSK2, rendering a common ancestry highly unlikely. Seven patients with splice-site alterations represented sporadic cases, and one patient represented a familial case. In three sporadic cases for which DNA samples were available from the mothers of the probands, including female patient CN49, we could demonstrate that the mutation arose de novo.

In five patients, small deletions or insertions resulted in a frameshift at the mRNA level (table 4); these included three distinct 1-bp deletions (in exons 4 and 6), a single 1-bp insertion (in exon 11), and a 5-bp deletion (in exon 17). All these frameshift mutations, predicted to produce a premature stop codon, were unique.

Seven nonsense mutations were characterized, in two

Table 3

Primers for Mutation Analysis

EXON	PRIMER SEQUENCE		LENGTH (bp)	ANNEALING TEMPERATURE (°C)
	Forward	Reverse		
2	TGATGTGAGGTCTGTTTTGGT	TTCATTTATCTATACCCTTTGTG	145	55
3	TTTGATTTTTAGCGGGAG	AAGACTGGTAGCATTTCATAA	285	55
4	GCAGGGTCTTATTTTAACAA	GACCTTTCAGTTTGTTTAGAC	210	55
5	AGTGGATATTACAATGTAGCA	GGGAGACGGCTCATACTTA	140	55
6	TATGAATGACCTAATGTAAACC	CAGGATGCATGTAAATAGACT	190	55
7	ACAGACACAAAAAGAAAATAAAT	ATCATATTACATTGTATTCAACT	200	50
8	GGATGTCAITTCAGTCTTA	GCCCTAATACAAGTTTTT	240	52
9	GATTAATGTCAGAACTCAAAG	ACAATCTCCTTCCCCTC	265	54
10	ACACTTCTGATTATTTTGCCT	CATCAAAACATCTATATTAAGGG	130	57
11	CCACCACAAAACAAATATCTACT	AAAATAAAAATACAGATGCCTCC	230	61
12	TTTCAAGCAAGTTTTTATTCT	GAAATAAAATTATCAGTGAATCC	125	57
13	GTATAGAATGAAAACCTTGCTTG	TATTTGTTGCTTATATTTGGAT	220	55
14	CTTCTTTGTCACCTTCTTACTC	TATCTTACAACATTCCAAATC	230	52
15	GGCATTATGGGACTCTTCCAC	GGCACTTTTAACAACAAGGGG	260	61
16	TGTATAGTGTGATGAGGTTT	AGACAACCTGATTCAAATGA	170	47
17	GTATATGATGATGGAAAGTAT	GTGTGTATGAAGATATAGAGTGG	190	56
18	AGGTCAGCACTCATCATC	TAAAACATACTAATACTGCAA	315	52
19	CAACTCATAGCCTTACAAATA	AAATGCTTAGGTGCTTAGAAC	180	55
20	TTTGATAGGAAGTGATACT	GAGTACTTTTGAGATTACC	210	47
21	AAGAGCCATAGAAAAAGAAGC	TGGAGGACCTGTGGAAAAC	245	60
22	TGTGTACGTGTGACTATCCATT	GTGTGCTTGCAGGTGTCTCTC	280	60

familial cases and five sporadic cases (table 4); two of these nonsense mutations were found in exon 5, affecting two neighboring codons (R112X and R110X), and one each of the remaining five were found in codons 10, 11, 17, 18, and 20. Six of these mutations occurred at CpG dinucleotides, affecting, in each case, an arginine codon (R110X, R112X, R233X, R305X, R514X, and R558X).

Although all these changes were unique in this series of patients, the R558X mutation observed in individual DZ34 already had previously been found in one patient, AH72 (Merienne et al. 1998). Haplotype analysis of the two patients DZ34 and AH72 (from France and Belgium, respectively) carrying the R558X mutations confirmed the presence of recurrent independent mutations.

Five missense changes, which are likely to be pathogenic, have been detected in the present study. The first N-terminal missense mutation, located in exon 4, resulted in the substitution of a phenylalanine for a valine at position 82. The altered valine is situated one residue beyond the putative ATP-binding site of the NH₂-terminal kinase domain and is highly conserved in all known protein kinases (Hank and Hunter 1995). Unfortunately, no clinical data were available for the male patient, AG89, bearing this mutation. An exchange of a glutamine for a histidine at position 127 (exon 5) was found in patient DX36, a sporadic male case. This histidine is highly conserved, not only in all RSK members but also in the majority of the known protein kinases (Hanks and Hunter 1995). The mother of this patient did not carry the mutation. A missense mutation ob-

served in exon 6 in a manifesting female patient, DX84, involved a substitution of tyrosine for aspartate at codon 154. This aspartate residue is highly conserved within most of the protein kinases (Hanks and Hunter 1995). The substitution A225V, in exon 9, was observed in one sporadic case, DG72. Alanine 225 is located two residues upstream of serine 227, which has been shown to be a phosphorylation site critical for RSK2 catalytic function (Fisher and Blenis 1996). In addition, protein-database comparison showed that alanine 225 is a conserved residue present in all known RSKs, including the homologues identified in *Drosophila* and *Xenopus laevis*. No clinical data were available for patient DG72, who carried this mutation.

Finally, a substitution of aspartate for glycine at position 431 was observed in patient BC72. Glycine 431 is located within the putative ATP-binding site of the C-terminal kinase domain and is conserved in all known protein kinases (Hanks and Hunter 1995). The clinically normal mother of this sporadic case did not carry the mutation.

We tested for the presence of these amino acid changes on ≥ 100 normal chromosomes, under the same SSCP conditions, and did not find them. This further supports the conclusion that these alterations are indeed disease causing.

Finally, two additional sequence changes were observed in the present study. A T→G transversion in exon 2, leading to the substitution of a nonpolar isoleucine for a polar serine at codon 38, was found in five unrelated CLS patients. The same change was found once

Table 4**RSK2 Mutations**

Patient (Sex)	Type of Mutation ^a	Referring Clinician(s)	Exon	Nucleotide Change	Predicted Protein or Splicing Alteration	Status of Mother
DX12 (M)	Sporadic	Schellely	4	del296T	Frameshift	Noncarrier
DZ15 (M)	Sporadic	Bay/Sell	4	del262A	Frameshift	Not tested
AG89 (M)	Familial	Pembrey	4	244G→T	V82F	Not tested
DZ28 (M)	Sporadic	Kirkpatrick	4	244 -2A→G	Destroy acceptor splice site	Not tested
DX36 (M)	Sporadic	Brookshire	5	379T→G	H127Q	Noncarrier
DZ26 (M)	Sporadic	Kress	5	328C→T	R110X	Carrier
BL4 (M)	Familial	Friedman/Berkenstadt	5	332C→T	R112X	Not tested
CN49 (F)	Sporadic	Kimberly	5	326 -1G→C	Destroy acceptor splice site	Noncarrier
DZ11 (M)		Storm/Willems	6	del451A	Frameshift	Not tested
DX84 (F)	Sporadic	Super	6	458G→T	D154Y	Noncarrier
AS93 (M)	Sporadic	Winter	6	486 +1G→A	Destroy donor splice site	Carrier
DG72 (M)	Sporadic	Tariverdian	9	674C→T	A225V	Noncarrier
DZ12 (M)	Sporadic	Mills	9	632 -2A→G	Destroy acceptor splice site	Not tested
DZ68 (F)	Sporadic	Crow	9	632 -1G→T	Destroy acceptor splice site	Not tested
DZ50 (M)	Sporadic	Torrado	10	817C→T	R223X	Not tested
DZ20 (M)	Sporadic	Stewart	11	ins910T	Frameshift	Not tested
DZ47 (M)	Sporadic	Graf/Ptacek	11	913C→T	R305X	Not tested
DZ3 (M)	Sporadic	Schinzl	13	1000 -2A→G	Destroy acceptor splice site	Noncarrier
BC55 (M)	Sporadic	David	14	1103 -1G→A	Destroy acceptor splice site	Noncarrier
BC72 (M)	Sporadic	Poncin	15	1289G→A	G431D	Noncarrier
DZ67 (M)		Crow	17	del1587TGAAA	Frameshift	Not tested
CU94 (M)	Sporadic	McDonald-McGinn	17	1540C→T	R514X	Carrier
DZ34 (M)	Familial	Delobel	18	1672C→T	R558X	Not tested
BG49 (M)	Familial	Berkenstadt	18	1764 +1G→A	Destroy donor splice site	Not tested
DX56 (M)	Sporadic	Kress	20	1934G→A	W645X	Not tested

^a For definition of sporadic cases, see Patients and Methods.

in 100 control chromosomes, suggesting that the variant is a likely nonpathogenic polymorphism. Finally, a CTA→CTC sequence variant at codon 266, which predicts no amino acid changes, was detected in 10 unrelated patients. Both of these changes have been previously reported, in non-CLS patients (Bjorbaek et al. 1995).

Discussion

To enable the full range of RSK2 mutations to be identified, we have determined the genomic organization and intron-exon boundaries of the human RSK2 gene. Our preliminary results show that ≥ 2 kb of the 3' UTR are present in exon 22 and suggest that the whole 5' UTR is present in exon 1. However, further studies on additional genomic and cDNA clones are necessary to complete this analysis. The RSK2 gene was screened for mutations, by means of SSCP analysis followed by direct sequencing of variant bands. We were unable to specifically amplify the first 70 bp of the coding region from exon 1, although several attempts were made with different primer pairs and amplification conditions. We suspect that the high GC-rich content of the region immediately upstream of the ATG translation-initiation site, in combination with the AT-rich composition of the first intron, generates conditions unfavorable for effi-

cient PCR. Tests with different polymerases, PCR conditions, and primers are ongoing.

In the analysis of 37 patients with clinical features suggestive of CLS, 25 distinct mutations were found, including 8 splice-site alterations, 7 nonsense mutations, 5 frameshift mutations, and 5 missense mutations. All were unique to this series of patients, and only two (R558X and 326 -1G→C) had been documented elsewhere, both in one family (Trivier et al. 1996; Merienne et al. 1998).

The seven nonsense mutations and the five insertion/deletion mutations are all expected to produce non-functional truncated proteins. The same holds true for six of the seven splice-site alterations. All these latter mutations hit either the conserved GT splice-donor dinucleotides or the conserved AG splice-acceptor dinucleotides and should thus lead to either exon skipping or activation of a cryptic splice site. Skipping is predicted to lead, in all but one case (326 -1G→C), to a frameshift in the misspliced exon sequences, resulting in protein truncation. The 326 -1G→C was shown to result in in-phase skipping of exon 5 in a previously reported patient carrying the same mutation. However, western blot analysis, using an antibody directed against the RSK2 C-terminus, failed to reveal RSK2 in this patient, which strongly suggests that the resulting internally deleted protein is unstable (Merienne et al. 1998). Clinical data

suggest that the extent of protein truncations does not correlate with the severity of the phenotype. Indeed, patients with various truncating mutations, including a 4-bp insertion (2105insGTGC) at the very 3' end and a stop mutation in exon 5 (R112X), show very similar severe mental retardation and skeletal changes. We speculate that truncated proteins are unstable, although this cannot be assessed, since no antibody for the N-terminal region is available.

Five missense changes, which are likely to be pathogenic, have been detected in this study. The glycine residue at position 431, located within the C-terminal kinase domain, is important for ATP binding (Hanks and Hunter 1995), and Valine 82 is located two residues downstream of the N-terminal ATP-binding motif. Fisher and Blenis (1996) have identified the N-terminal domain as being responsible for substrate phosphorylation and have shown that the N-terminal domain is necessary for autophosphorylation and, thus, for optimal enzyme activity. Therefore, both the G431D mutation and the V82F mutation are predicted to have detrimental effects on the transduction function of RSK2. Unfortunately, no cell lines were available to test the RSK2 kinase activity in the patients with these mutations. However, we have shown elsewhere that the substitution of another highly conserved glycine residue from the N-terminal ATP-binding site (G75V) dramatically impairs RSK2 phosphotransferase activity (Trivier et al. 1996). The A225V missense is two residues upstream of an autophosphorylation (serine 227) site important for maximal enzyme activity (Zhao et al. 1995). Finally, two missense mutations in exons 5 and 6, H127Q and D154Y, affect residues conserved in all protein kinases, suggesting an important functional role. In addition, both mutations were shown to occur as *de novo* mutations. The biochemical effect of these two mutations is less evident, since they occur in protein domains with no predicted function. However, it could be hypothesized that a conformational change affects the protein catalytic function, since, in both cases, a charged amino acid is replaced by a polar noncharged residue in the N-terminal kinase domain. It would be of interest to study the effect that these missense mutations have on the biochemical activity of RSK2.

Two additional sequence changes were observed in the present study, including one amino acid change (S38I) and one neutral nucleotide substitution (A798C). These appear to be polymorphisms in RSK2, rather than disease-causing mutations.

We have screened 21 exons covering 97% of the RSK2 coding sequence and have found mutations in only 25 patients. Obviously, it is of importance to know whether patients negative for a mutation might represent heterogeneity. However, for the present study there was no formal clinical consensus on patients before they were

analyzed. As a result, available clinical data were often incomplete or even lacking. In addition, photographs have been obtained, to date, only for a few patients. Therefore, and also because some of the patients with no identified mutation may be mutated in regions of the RSK2 gene that have not yet been explored (e.g., in exon 1, in introns away from exon-intron boundaries, or in regulatory elements), the question of heterogeneity was not examined in this study and, thus, remains open. To address this question, further studies (as a first step, the screening exon 1 of the RSK2 gene and the collection of additional clinical data) are necessary. To this end, a detailed questionnaire for standardized clinical data is now systematically sent to the clinicians. Finally, it is of interest to note that we found three mutations in female probands who were ascertained through learning disability and mild but suggestive physical phenotype but who had no affected male relatives.

Results from the present study, coupled with data on previously reported mutations (Trivier et al. 1996; Merienne et al. 1998), bring the total of different mutations to 34, illustrating allelic heterogeneity (fig. 1); the vast majority are point mutations, with only 6 (16%) occurring in CpG dinucleotides in the coding region of the gene; there is a total of 8 amino acid substitutions (22%), which may be of interest for functional studies. Mutations are quite evenly distributed throughout the gene, with no obvious clustering. However, one-third of mutations were found in exons 4, 5, and 9, and no mutations have been found yet in exons 2, 8, 12, 16, 19, and 21. Thirty percent of the mutations involve consensus splice sites, a rate higher than the 15% observed among all point mutations causing human diseases (Krawczak et al. 1992). To date, in only one patient was the mutation shown to be a large deletion detected by Southern blot analysis (Trivier et al. 1996).

We showed that the mutation arose *de novo* in 11 (68%) of the 16 sporadic cases for which samples were available from the mothers of the probands (table 4). This rate of new mutations is twice that expected (33%) for a severe X-linked disease in which male patients do not reproduce. This may be explained either by a higher mutation rate during oogenesis versus spermatogenesis or, more likely (given mutation heterogeneity), by a decreased reproductive fitness in carrier females. However, although there was no obvious selection bias in the mothers studied, this result needs to be confirmed in additional families.

Some clinical data were available from 17 patients with an identified mutation (3 had amino acid substitutions, and 14 had truncating mutations). No consistent relationship was observed between the severity of clinical phenotype and the type of the RSK2 mutations. This may be due to the fact that all these mutations cause a loss of function. Also, no obvious correlation was found

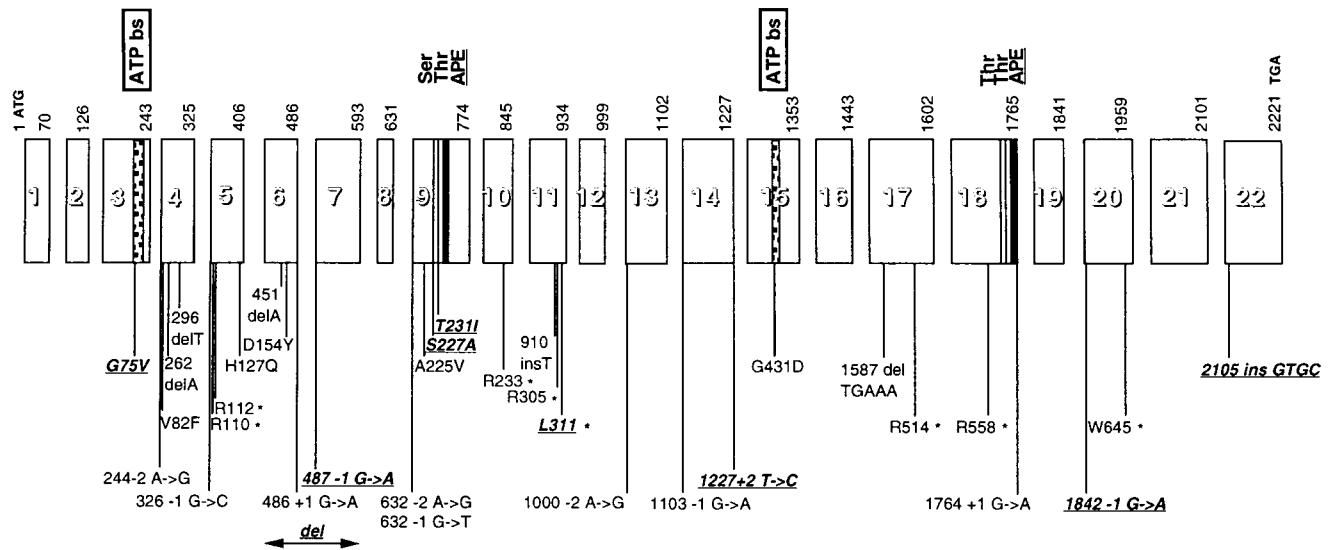


Figure 1 Intron-exon structure of the human Rsk2 gene and mutations in CLS. Exons are represented as boxes and numbered “1”–“22.” The last nucleotide position of each exon is indicated at the top. Both ATP-binding sites (ATP bs) are shown as stippled boxes (in exons 3 and 15). Within exons 9 and 18 are represented the alanine-proline-glutamine (APE) motifs, as blackened boxes, and the putative phosphorylation sites (Ser and Thr), as lines. The different mutations detected are shown schematically below the gene. Mutations that have been published previously (Trivier et al. 1996; Merienne et al. 1998) are underlined.

between specific mutations and the expression of particular features. For example, three patients with nonsense mutations and one patient with a missense mutation (S227A in patient AG96, previously reported by Trivier et al. 1996) had bilateral hearing loss, whereas no hearing defect was documented in the remaining patients. Another feature, hyperplexia (attacks consisting of stimulus-sensitive falling), was documented only in three male patients carrying different truncating mutations. Moreover, the phenotype of patients DZ34 (of the present study) and AH72 (of the study by Trivier et al. 1996), who share a nonsense mutation (R558X), was shown to vary considerably. Patient DZ34 (at the age of 17 years) had severe kyphoscoliosis and a bilateral hearing deficit, whereas patient AH72 (at the age of 22 years) had no hearing deficit and no kyphoscoliosis. Although preliminary, these observations strongly suggest that other environmental or genetic factors influence the phenotypic severity of CLS.

CLS shows marked phenotypic variability and a high rate of sporadic cases (~70% of the patients with CLS in our series of families with an identified mutation). These factors can lead to diagnostic—and, hence, counseling—difficulties. The identification of the CLS gene now makes accurate post- and prenatal diagnosis in families possible. However, the widespread dispersion of mutations and the rather large number of exons will not facilitate mutation testing. Since most RSK2 mutations lead to premature termination of translation and/or to

loss of phosphotransferase activity, we have been developing immunoblot and RSK2 kinase assays as rapid (1 d) and simple diagnostic tests for CLS. Immunoblot analysis has recently allowed us to confirm the clinical diagnosis of CLS in 6 of 13 patients and to confirm the results of the kinase assay in 1 additional patient; mutations in the RSK2 gene subsequently have been found in 5 of these patients (Merienne et al. 1998).

The study of mutations in RSK2 and of their relationship to the CLS phenotype is just beginning. Further investigations will clarify the physiological function of RSK2 and are likely to result in a deeper understanding of this disorder.

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Electronic-Database Information

Accession number and URL for data in this article are as follows:

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