

Rapid immunoblot and kinase assay tests for a syndromal form of X linked mental retardation: Coffin-Lowry syndrome

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Abstract

Coffin-Lowry syndrome (CLS) is a syndromal form of X linked mental retardation, in which some associated facial, hand, and skeletal abnormalities are diagnostic features. Accurate diagnosis, critical for genetic counselling, is often difficult, especially in early childhood. We have recently shown that Coffin-Lowry syndrome is caused by mutations in the gene encoding RSK2, a growth factor regulated protein kinase. RSK2 mutations are very heterogeneous and most of them lead to premature termination of translation or to loss of phosphotransferase activity or both. In the present study, we have evaluated immunoblot and RSK2 kinase assays as a rapid and simple diagnostic test for CLS, using cultured lymphoblastoid or fibroblast cell lines. Western blot analysis failed to detect RSK2 in six patients, suggesting the presence of truncated proteins in these patients. This conclusion was confirmed in four patients, in whom the causative mutations, all leading to premature termination of translation, were identified. Of four patients showing a normal amount of RSK2 protein on western blot and tested for RSK2 phosphotransferase activity, one had a dramatically impaired activity. Analysis of the RSK2 cDNA sequence in this patient showed a mutation of a putative phosphorylation site that would be critical for RSK2 activity. Preliminary results show that, at least, the western blot protocol can be successfully applied to lymphocyte protein extracts prepared directly from blood samples. These assays promise to become important diagnostic tools for CLS, particularly with regard to very young patients with no family history of the condition.

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Coffin-Lowry syndrome is an X linked syndrome (CLS) (MIM 303600) characterised by severe psychomotor retardation and typical facial, hand, and skeletal malformations. In male patients over 5-10 years of age, the disease has a well defined phenotype and, in spite of some variability, can in most cases easily be diagnosed by professionals trained in genetic

disorders. Typically they are of short stature and exhibit a characteristic coarse face with a prominent forehead, orbital hypertelorism, downward slanting palpebral fissures, epicanthic folds, thick lips, a thick nasal septum with anteverted nares, and irregular or missing teeth. Their large and soft hands with lax skin and tapering fingers are usually a strong diagnostic feature. The most frequent skeletal changes are delayed bone development, spinal kyphosis/scoliosis, and pectus carinatum or excavatum.¹⁻⁶ Less constant features include microcephaly, ventricular dilatation, sensorineural deafness, cardiac defects, and secondary sexual characteristics.^{5,7} The occurrence of seizures has been reported in a few cases.⁸ Mental retardation may be highly variable, but most male patients appear to be severely affected. Several authors have suggested that it is a progressive disorder since retardation of growth and psychomotor development appear gradually in the first years of life.^{1,3,5,9} Furthermore, facial coarsening and skeletal involvement become more pronounced with age. Coffin-Lowry syndrome is usually considered a rare condition, but its prevalence may be underestimated owing to lack of ascertainment and failure to diagnose milder or atypical cases.

Establishing the diagnosis in very young male patients (below 5-10 years of age) is often much more difficult than in older patients, since the physical characteristics are usually mild and can be confused with other syndromes, most notably mental retardation with fragile X (MIM 309550), Sotos syndrome (MIM 117550), Williams syndrome (MIM 194050), and α thalassaemia with mental retardation syndrome (ATR-X, MIM 300032). Furthermore, young ATR-X patients showing clinical features highly reminiscent of Coffin-Lowry syndrome and without α thalassaemia have been reported.¹⁰ Tapering fingers, the most reliable diagnostic feature in infancy, may also be observed in other mental retardation syndromes, including Borjeson syndrome (MIM 301900) and Prader-Willi syndrome (MIM 176270). An additional complication arises from the high rate of sporadic cases, approximately 60% in our series of 80 families.

Early diagnosis of Coffin-Lowry syndrome is essential for proper management of the patients, including prevention of skeletal complications, and for genetic counselling. With the recent identification of the gene defective in

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CLS patients, DNA based genetic testing has become feasible.¹¹ The gene codes for RSK2, a kinase implicated in the activation of the mitogen activated kinase cascade and the stimulation of cell proliferation and differentiation.¹² RSK2 transcripts are present in a wide variety of adult tissues, including fibroblasts and lymphocytes, which have the highest levels.¹³ The genomic structure of the gene has not yet been completely elucidated, but our current results indicate that the 2200 base pairs of the RSK2 transcript are organised into at least 22 exons. While Southern blot screening of over 60 patients has shown only one intragenic RSK2 deletion, SSCP analyses have identified to date 20 point mutations and one 4 bp insertion in CLS patients¹¹ (S Jacquot, in preparation). All are unique and evenly distributed over the entire coding sequence. These results, although preliminary, support considerable molecular heterogeneity in CLS. The identification of an RSK2 mutation provides definitive confirmation of Coffin-Lowry syndrome. However, given the large size and complex structure of the RSK2 gene, and the diversity of the mutations, systematic search for mutations is very time consuming. Therefore, rapid and accurate prescreening tests would be of value in the differential diagnosis of CLS. Our current data show that about 65% (14 of 22 to date) of the RSK2 lesions result in truncated RSK2, lacking various parts of the C-terminus. As shown previously, these mutations can readily be detected in affected males by the absence of a signal on western blot with the commercially available anti-RSK2 antibody, which is directed against the C-terminus of RSK2.¹¹ We have also already shown that at least some missense mutations, those affecting phosphorylation or ATP binding sites, impair the RSK2 phosphotransferase activity when tested in an in vitro S6 kinase assay.¹¹ Thus, in the present study we have evaluated western blotting and S6 kinase assays as rapid prescreening tests for RSK2 mutations.

Patients and methods

A series of 13 unrelated males, with ages ranging from 4 to 20 years and clinically diagnosed as having CLS, were investigated in the present study. They had been referred to us for genetic studies by European, American, or Australian clinicians, and were mostly sporadic cases (10/13). Four patients, AB21, AG96, AZ21, and DX34, whose diagnosis had previously been confirmed by the identification of a mutation in the RSK2 gene,¹¹ were included in the study as positive or negative controls. Lymphoblast or fibroblast cell lines derived from the patients were established by the Genethon or by the Laboratoire de Génétique, CHU Nancy. Control lymphoblastoid cell lines or blood samples from healthy subjects were also included in the study.

Total protein extractions from cell lines and western blot analysis were performed as previously described.¹¹ Before western blotting, an immunoprecipitation step was introduced in order to increase the concentra-

tion of RSK2 protein. Immunoprecipitation was carried out with a goat polyclonal antibody specific for RSK2 (C-19) (Santa Cruz Biotechnology) and, after blotting, membranes were incubated with the same antibody. This protocol was also applied to the same samples using an antibody specific for RSK3 (antibody C-20), which was used as internal control.

Blood samples (10 ml) were loaded on histopaque-1077 (Sigma) gradients to isolate lymphocytes. Protein extraction from lymphocytes and western blot analysis were as for cell lines.

Kinase assays were performed as described previously,¹¹ using protein extracts immunoprecipitated with C-19 anti-RSK2 antibody and the S6 kinase assay kit distributed by Upstate Biotechnology.

Results

The goat anti-RSK2 polyclonal antibody C-19 (Santa Cruz Biotechnology), initially tested by western blot analysis on protein extracts from lymphoblastoid cell lines of eight normal subjects, showed a band of the expected size (approximately 90 kb) in all of them (not shown). Since C-19 antibody showed slight cross reaction with other RSK members, an immunoprecipitation step was introduced before western blotting in all subsequent studies of patients. Addition of this immunoprecipitation step increased the intensity of the RSK2 signal. However, the different RSK members having different molecular weights (and RSK2 the highest), this step can be omitted, leading to a more rapid test, with results remaining interpretable.

Table 1 Results in Coffin-Lowry syndrome patients. Column A: presence (+) or absence (-) of the full length protein RSK2 as detected by western blotting. Column B: the corresponding mutation when it was determined. Numbering for splice site and insertion mutations refer to nucleotide positions and for missense and nonsense mutations to codon positions. The first four patients, AB21, AG96, AZ21, and DX34, have been characterised previously and are used in the present study as controls. (L) and (F) in the first column refer respectively to the lymphoblast or fibroblast cell lines established from the corresponding patient

Patient	Age (y)	A: RSK2 protein	B: RSK2 mutation
AB21 (L)		-	Splice site 1842-1 G→A
AG96 (L)		+	Missense S227A
AZ21 (L)		-	Deletion Δ (406-593)
DX34 (F)		-	Splice site 1227+2 T→C
BB89 (L)	15	-	Splice site 326-1 G→C
BQ75 (L)	6	-	Nonsense L311X
CE21 (L)		-	NT
CV59 (F)	4	-	NT
DD39 (L)	9	+	NT
DX6 (L)	14	+	Missense T231I
DX9 (L)	9	+	NT
DX15 (L)	13	+	NT
DX29 (L)	19	+	NT
DX55 (L)	20	+	NT
DX57 (L)	6	-	Splice site 487-1 G→A 4 bp insertion 2104+GTGC
DX88 (L)	16	-	NT
DZ27 (F)	8	+	NT

NT: not tested.

Western blot analysis, using C-19 antibody, failed to show RSK2 in six patients, whereas a band of the same size and similar intensity as in normal controls was observed in the remaining seven patients (table 1). RSK3 protein was present at roughly comparable levels in all patients and controls. Fig 1A illustrates results for some patients. In extract AZ21, RSK3 but not RSK2 was detected, as expected from our previous study.¹¹ In this patient, a large RSK2 intragenic deletion was previously found resulting in a truncated gene protein product, which lacks the epitope recognised by C-19 anti-RSK2 antibody.¹¹ Direct sequencing of RT-PCR products, amplified as previously described,¹¹ from mRNA purified from cell lines of four other patients (DX57, BB89, DX88, and BQ75) lacking RSK2, showed the causative mutation in all of them: two splice site mutations resulting in frameshifts (DX57 and BB89), a nonsense mutation (BQ75), and a 4 bp insertion (DX88) (table 1). These mutations lead to truncated RSK2 proteins, lacking various parts of the C-terminus end, including in all four patients the epitope recognised by the C-19 antibody.

A band corresponding to the RSK2 protein was also detected by western blotting, using the C-19 antibody and proteins extracted from 10 ml blood samples from six normal subjects (one representative result is shown in fig 1B).

In this experiment, proteins were also immunoprecipitated with the C-19 antibody before western blot analysis. Thus, this result indicates that RSK2 protein is readily detected using lymphocyte protein extracts prepared directly from blood samples.

We then tested the possibility that some of the patients in whom RSK2 is present carry missense mutations affecting the RSK2 phosphotransferase activity. In addition to the negative control AZ21¹¹ and a normal control, cell lines from four patients (DX6, DX9, DX15, and DD39) were assayed. As shown in fig 1C, S6 substrate was readily phosphorylated by RSK2 from three patients (DX9, DX15, and DD39), while that from patient DX6 had strikingly impaired kinase activity, comparable to the activity of the negative control patient AZ21 (not shown). Sequence analysis of RT-PCR products amplified from the lymphoblastoid cell line from patient DX6 showed only one change when compared to the normal RSK2 sequence,¹⁴ a T to C transition at base 692, resulting in a threonine to isoleucine substitution at codon 231. Threonine 231 is located five amino acids upstream of the highly conserved A(S)PE motif present in all protein kinases, within a region known often to contain phosphorylation sites.¹⁵ Thus, this result strongly suggests that threonine 231 is a residue critical for catalytic function.

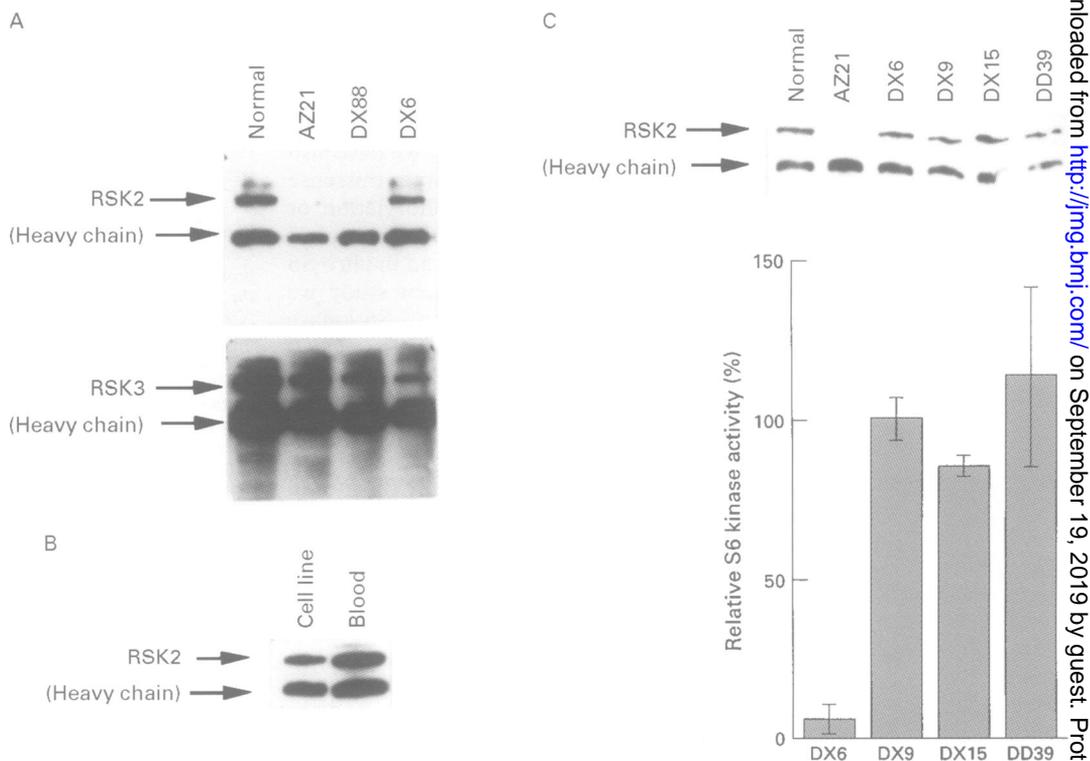


Figure 1 Western analysis and enzymatic activity of immunoprecipitated RSK2 protein from CLS patients. (A) Western analysis of immunoprecipitated RSK2 (top) and RSK3 (bottom) proteins from equivalent amounts of total proteins. The origin of the lymphoblastoid cell lines is indicated. (B) Western analysis of immunoprecipitated RSK2 proteins from a blood sample and a lymphoblastoid cell line (both from normal subjects). In the experiment shown, double the amount of total protein (6 mg) was immunoprecipitated from the blood sample than from the lymphoblastoid cell line. (C) RSK2 phosphotransferase activity of immunoprecipitated proteins from CLS diagnosed patients. Top, western analysis of immunoprecipitated RSK2 proteins (from equivalent amounts of total proteins for each lane). Bottom, S6 kinase activity of the same immunoprecipitated proteins. Data are represented as a percentage of the specific RSK2 activity immunoprecipitated from various wild type cell lines (the wild type relative specific activity being 100% \pm 5.6). 0% was set for the residual kinase activity determined from the negative control AZ21 cell line. Owing to the presence of contaminating kinase activities in the immunoprecipitated protein extract, this non-specific residual activity reached 25% \pm 3.7 of the wild type activity. Origin of the lymphoblastoid cell lines is indicated. The immunoglobulin heavy chain (immunoprecipitation) is shown by western blotting. The light chain has migrated out of the gels.

According to the results shown in fig 1C, either the diagnosis of CLS of DX9, DX15, and DD39 is incorrect, or these patients carry mutations that do not affect the phosphotransferase activity of the enzyme, which does not exclude that another function of the protein is deficient (regulation, cellular localisation). In order to elucidate this point, we are currently searching for mutations located in the RSK2 gene of patients DX9, DX15, and DD39.

Discussion

This study was designed to evaluate the feasibility of using western blot analysis in combination with kinase assays as prescreening tests for RSK2 genetic defects on a routine basis. As a preliminary step, the reliability of the polyclonal C-19 anti-RSK2 antibody for this study was tested on normal controls. Our results, although based on a limited number of samples (eight cell lines and six blood samples), showed that the RSK2 protein is consistently and easily detected in normal subjects by the C-19 antibody. Western blotting results obtained using patients' derived cell lines showed that the diagnosis of CLS can be confirmed very rapidly, within one day, in a significant proportion of patients (table 1). Indeed, of 13 clinically CLS classified patients, RSK2 was not detected in six cases. Four of them were tested by sequencing of the cDNA, and a mutation was identified in the RSK2 gene. Predicted products of these mutant genes are various truncated RSK2 proteins, all lacking the epitope recognised by the anti-RSK2 antibody (table 1). These further results confirm both the western blot data and the diagnosis of Coffin-Lowry syndrome in these patients. Thus, western blot analysis alone should allow confirmation of approximately half of clinically diagnosed CLS patients. These experiments were performed on cell line extracts. However, the procedure based on western blotting may be accelerated by using protein extracts prepared directly from blood samples (less than four days old) as shown in fig 1B.

A second step that may be introduced in the prescreening test consists of assaying the kinase activity of RSK2 in patients in whom western blot analysis shows a full length RSK2 protein. In this study, the phosphotransferase activity of RSK2 was tested for four of the seven patients who fulfil this latter condition. RSK2 protein of patient DX6 has a catalytic activity that is dramatically affected, indicating that for this patient the diagnosis of CLS is correct. This conclusion was confirmed by the finding of a missense mutation (T231I) located within the catalytic pocket of the N-terminus enzymatic domain. The latter was shown to be directly involved in the phosphotransferase activity of the kinase.^{14,16} The mutated threonine is assumed to be a target for a phosphorylation that is necessary for the kinase activity. Further studies are warranted to confirm this point. The second step of the prescreening test, based on a functional assay, appears thus to be useful for confirming some additional diagnoses. In

the remaining three patients where a S6 phosphotransferase activity (fig 1C) in the normal range was found, the diagnosis of CLS can neither be confirmed nor excluded. The diagnosis may be incorrect, though they all show typical features of Coffin-Lowry syndrome, or a function different from the catalytic function of the kinase may be altered. For instance, domains involved in regulation or in nuclear targeting may be affected by a mutation. Indeed, such mutants would be of interest for a more comprehensive understanding of the disease and of the cellular function of the RSK2 kinase. We are currently searching for mutations in the RSK2 gene in these three patients.

It is noteworthy that all the mutations leading to premature termination, as well as the missense mutations abolishing phosphotransferase activity (for instance in patient AG96¹¹ and in patient DX6) so far identified, are constantly associated with typical and severe CLS phenotypes, including mental impairment. Our present results suggest that CLS patients likely to be detected through these tests may be those developing the most severe form. In females, RSK2 is expected to be expressed only from the active X chromosome. Thus, owing to random X inactivation, it is likely that expression of the normal gene protein product is highly variable in heterozygous females and that carrier detection may not be feasible by these assays. Studies are under way to investigate this point.

Both assays used in this study are simple, reliable, and rapid methods and should become important diagnostic tools for CLS, particularly for very young patients with no family history of the condition. Preliminary data show that at least the western blot analysis can be carried out directly on leucocytes from blood samples. Studies to validate further this latter protocol, as well as a kinase activity test directly from a blood sample, are in progress.

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