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Diamond Blackfan anaemia in the UK: clinical and genetic heterogeneity

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Summary

A detailed family study was undertaken of patients notified to the UK Diamond Blackfan Anaemia (DBA) Registry. *RPS19* mutations were detected in 16 of 104 families, including two patients with deletions detected by intragenic loss of heterozygosity of tightly linked polymorphisms. In two further cases, polymorphisms were used to determine the parental allele of origin of *RPS19* point mutations. A review of clinical details of patients with mutations and patients in the literature having identical or equivalent mutations revealed evidence for a genotype:phenotype correlation with respect to the prevalence of physical anomalies, and the occurrence of mild or variable haematological severity. Nine of 60 patients had a known family history of DBA. Haematological abnormalities, including raised red cell adenosine deaminase activity, were found in first-degree relatives of 16 of 51 (31%) of patients not previously considered to have familial DBA. Results of both parents and any siblings were normal in only 35 of 60 (58%) of cases, who were therefore assumed to have sporadic *de novo* DBA. The classical inheritance pattern for DBA is autosomal dominant; however, 12 of 60 families (20%) had more than one affected child despite normal results in both parents. These results have important implications for genetic counselling, and for the selection of potential sibling bone marrow donors.

Keywords: Diamond Blackfan anaemia, *RPS19* mutations, polymorphism, genetic counselling, adenosine deaminase.

The UK Diamond Blackfan Anaemia (DBA) Registry was established with the aim of collating representative population-based data for studies on the aetiology, pathophysiology and treatment of this rare disorder. The initial analysis focused on the incidence, associated physical anomalies and response to treatment in a 20-year birth cohort, and was reported by Ball *et al* (1996). As then there have been significant advances in the genetics of DBA, most notably the identification of the gene encoding small ribosomal protein 19 (*RPS19*), as the first DBA gene (Draptchinskaia *et al*, 1999). This followed the serendipitous finding of a *de novo* balanced t(X;19) translocation disrupting *RPS19* in a patient with DBA (Gustavsson *et al*, 1997a). *RPS19* mutations have subsequently been described in up to 26% of patients with DBA in a European collaborative study (Willig *et al*, 1999). Linkage analysis has shown evidence for linkage to chromosome 8p in some families without *RPS19* mutations, but there are also families with DBA linking with neither 8p nor 19q, where *RPS19* is located, showing that there must be at least three genes responsible for DBA (Gazda *et al*, 2001).

There has also been an increasing recognition that the phenotypic spectrum of DBA is broader than that of the classical presentation of severe red cell aplasia presenting in early infancy (Alter & Young, 1998). An increase in red cell adenosine deaminase (eADA) activity may be the only manifestation of DBA (Willig *et al*, 1998). This has important implications for genetic linkage studies, in which the phenotypic definition of affected and non-affected family members is critical. The existence of a clinically silent DBA phenotype also presents a particular hazard in the exclusion of DBA in sibling bone marrow donors (Orfali *et al*, 1999; S. Howell, S. Ball, K. Orfali, A. Will, T. Carr, R. Stevens & R. Wynn, unpublished observations) and complicates genetic counselling. It has

become increasingly difficult to advise the parents of a baby with newly diagnosed DBA of the risk to future pregnancies, which has particular relevance for families hoping to select a future stem cell donor for their affected child by pre-implantation human leucocyte antigen typing.

We have therefore undertaken a detailed *RPS19* mutation and haematological analysis in a population-based study of UK DBA patients and their first-degree relatives. Our results confirm that the prevalence of familial DBA is significantly higher than previously reported, with haematological abnormalities detected in 31% of families of patients originally classified as having sporadic *de novo* DBA. In addition, in a significant proportion of families with inherited DBA the pattern of inheritance is not clear, with implications for both genetic counselling and genetic linkage studies.

Methods

Family study

Patients were identified from the UK registry, including the cohort previously described (Ball *et al*, 1996) and new families subsequently notified to the registry. Clinical details and family history were obtained from registry data, referring clinicians and information directly volunteered by participating families. Blood samples from probands and their first-degree relatives were collected into EDTA for full blood count, eADA assay and DNA extraction, following informed consent. Local ethical approval was obtained for the study.

Erythrocyte adenosine deaminase (eADA) assay

Erythrocyte adenosine deaminase activity was measured by a spectrophotometric assay (Agarwal & Parks, 1978), as described (Ball & Orfali, 2004). The normal range was determined by assaying eADA activity in samples from 44 normal controls, the upper limit being defined as the normal mean + 2 standard deviations (SD).

RPS19 mutation analysis

DNA was extracted using a genomic DNA purification kit (Promega, Southampton, UK) or by a standard proteinase K, phenol and chloroform method (Sambrook *et al*, 1989). Polymerase chain reaction (PCR) fragments encompassing the six exons and 450 bp 5′ UTR of RPS19 were amplified from genomic DNA using four primer sets, as previously described (Draptchinskaia *et al*, 1999; Willig *et al*, 1999; Ball & Orfali, 2004). PCR products were purified by agarose gel electrophoresis and QIAquick column purification (Qiagen, Crawley, UK). Sequencing was performed at the Advanced Biotechnology Centre (ABC), Imperial College, London by Applied Biosystems (Foster City, CA, USA) automated DNA sequencing technology, using the original PCR primers and additional internal primers as required. Sequence variations were verified by sequencing on both strands of a freshly amplified independent PCR product. When a mutation was identified in a proband, all available family members were screened for that mutation.

Cloning of PCR products for sequence analysis of individual alleles

To clarify the sequence of heterozygous insertion/deletion sequence variations, or to determine the parental allele of origin of *de novo* mutations, PCR products encompassing both the mutation and a potentially informative polymorphism were subcloned into a TA pCR® 2.1 vector (Invitrogen, Paisley, UK). Individual colonies were picked for expansion, then plasmid DNA was purified using a *Wizard® Plus* SV miniprep kit (Promega). The *RPS19* insert was amplified using the relevant primers for sequencing, as above.

Seasonality

The Edwards (Edwards, 1961; St Leger, 1976) and Kolmogorov–Smirnov (Freedman, 1979) tests for seasonality were applied as in the original registry analysis (Ball *et al*, 1996), using the UK Birth Statistics FM1 series to obtain data on normal monthly variation in birth figures.

Results

eADA activity in probands and first-degree relatives (Fig 1)

The distribution of eADA activity in 44 normal controls was used to establish the normal range, with the upper limit of normal of 1·00 U/gHb being defined as the mean + 2SD. eADA activity was above the normal range in 48 of

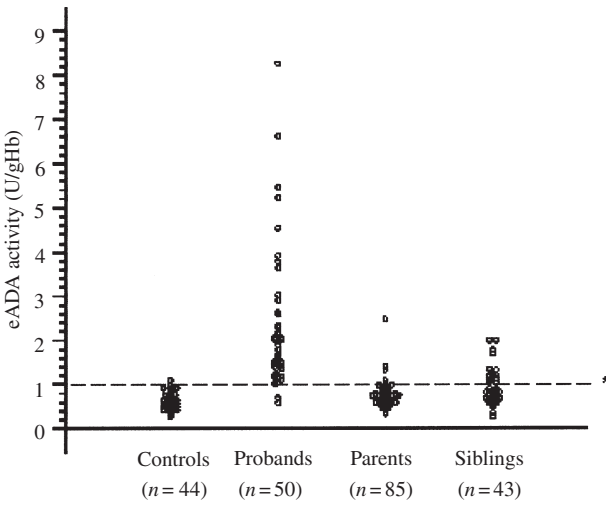

Fig 1. eADA activity in controls, transfusion-independent probands and their first-degree relatives. *Upper limit of normal 1·00 U/gHb (mean + 2 SD).

50 transfusion-independent patients with DBA (range 0·58–8·26 U/gHb; mean 2·27 U/gHb). eADA was also raised in 17 of 37 patients who were transfusion dependent (range 0·31–3·57 U/gHb; mean 1·03 U/gHb), the lower values reflecting the presence of transfused red cells with normal eADA activity. Eighty-five parents (45 mothers; 40 fathers) of children with DBA were studied. The majority of eADA activities were within the normal range, but six parents (7%) (three mothers; three fathers) had raised eADA activity. Twenty-two of the 43 siblings tested (51%) also had eADA activity above the normal range (Fig 1). In families with known familial DBA, first-degree relatives already known to have DBA were excluded from the parent or sibling groups.

Patterns of familial DBA and high eADA

Sixty families, for which data were complete, were subclassified according to the presence or absence of a known family history of DBA, and of haematological abnormalities in first-degree relatives, including isolated raised eADA.

Overt family history. Nine of the 60 families (15%) had a clear family history of DBA. In five, there was an autosomal dominant pattern of inheritance, although in one family the father of two affected half-siblings had normal results, despite being an obligate carrier. In four families, there were two affected siblings despite both parents having normal results [normal haemoglobin, mean cell volume (MCV) and eADA, and no history of anaemia]. There was no parental consanguinity.

Abnormalities revealed on family study. Haematological abnormalities were found in a further 16 of the 60 families (27%), which represented 31% of the 51 families in which familial DBA had not previously been diagnosed.

In nine cases, the family results were consistent with an autosomal dominant pattern of inheritance, with high eADA or anaemia in more than one generation, and in some cases also affecting siblings of the proband. In one family, the father and three children had a history of self-limiting anaemia in infancy, resembling transient erythroblastopenia of childhood (TEC). The father and oldest sibling had made a full haematological recovery, except for a persistently raised eADA activity. This would be an unusual finding in the more common non-familial TEC, in which normal eADA activity is a useful discriminant (Glader, 1987). In two families, there was a history of anaemia during pregnancy, which is known to exacerbate anaemia in DBA (Rijhsinghani & Wiechert, 1994; Alter *et al*, 1999). There was persisting mild anaemia and macrocytosis with high eADA activity in one case, and residual isolated high eADA activity in the other. In one family, both unrelated parents of a child with DBA had raised eADA, but normal haemoglobin and MCV, and no history of anaemia.

In a further seven families, the proband had one or more siblings with anaemia or raised eADA activity, despite both parents having completely normal results, including normal eADA activity. In one family, the proband's sister, who was haematologically normal at the time of study except for raised eADA activity, later developed profound steroid-responsive macrocytic anaemia (S. Howell, S. Ball, K. Orfali, A. Will, T. Carr, R. Stevens & R. Wynn, unpublished observations).

Normal family results. Sporadic *de novo* DBA was inferred in 35 families in which both parents and any siblings had completely normal results, including eADA. This represented 58% of all 60 families, or 69% of the 51 families without an overt family history of DBA.

Seasonality of birth month in sporadic DBA

The original report of UK DBA Registry had highlighted a possible seasonal pattern in the birth month of babies with sporadic DBA (Ball *et al*, 1996), with a broad autumn–winter peak, and an additional sharp peak in May. The results presented here show that the original study included a significant proportion of probands who could no longer be considered to have *de novo* DBA. Figure 2 shows the corrected birth month distribution for probands in the current study with normal family results, excluding one born outside the UK. The peak in May, which was observed in the previous study, was no longer apparent, but there was still an uneven birth month distribution, with a broad peak in the summer and autumn. This apparent birth month seasonality achieved statistical significance ($P < 0{\cdot}05$) with the Edwards test for seasonality (Edwards, 1961), referenced to percentage points modified for the small sample size (St Leger, 1976), but not when the Kolmogorov–Smirnov test (Freedman, 1979) was applied. However, given the now known genetic diversity of DBA, it is unlikely that the unequal distribution of birth month reflects anything other than the chance result of low numbers.

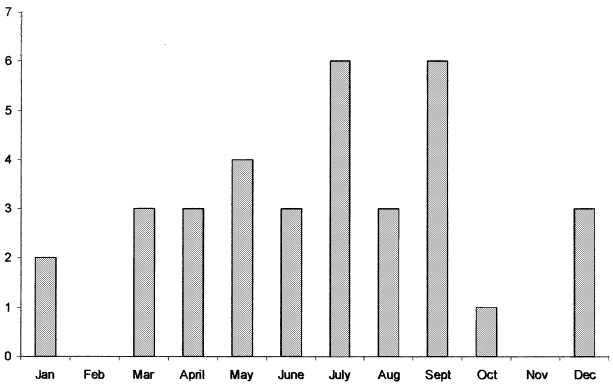

Fig 2. Birth month distribution of 34 probands with presumed sporadic Diamond Blackfan anaemia. Individuals born in the UK; both parents and all siblings tested have normal results including eADA.

Table I. Clinical data on probands in this study with *RPS19*.

Family	Mutation	Predicted effect	Inheritance	Parental origin
E3	tcagATG → tcatATG†‡	IVS1 acceptor G → T	Familial	Father and grandfather
E10	383/4 del AA‡	Frameshift codon 128	Familial	Father and grandmother
E13	250/251 del AG‡	Frameshift codon 84	Sporadic	Non-informative
E25	Intragenic LOH	Allele loss	Sporadic	Paternal
E28	182C → A‡	Ala61ser	Sporadic*	Non-informative
E49	34 C → T	Gln12stop	Sporadic	Paternal
E54	−633 ins GCCA	Effect on promoter?	nk	nt
E61	344/345 ins AA	Frameshift codon 116	nk	nt
E63	167 G → A	Arg56Gln	Sporadic	Paternal
E64	AAAgtgag → AAAatgag	IVS2 donor G → A	Sporadic	Non-informative
E71	280 C → T	Arg94Stop	nk	nt
E73	383/4 del AA	Frameshift codon 128	nk	nt
E75	Intragenic LOH	Allele loss	Sporadic	Paternal
E83	331 del C	Frameshift/stop codon 110	Sporadic	Non-informative
E85	169 G → C	Ala57Pro	Sporadic	Non-informative
E98	3 G → T	Met1Ile	Sporadic	Non-informative

del, deletion; ins, insertion; LOH, loss of heterozygosity; IVS, intervening sequence; nk: not known; nt: not tested.
RPS19 nomenclature: mRNA numbering from initiation codon for coding sequence mutations; mutations in 5′ non-coding region: nucleotides 5′ to initiation codon in genomic sequence.
Sporadic: *RPS19* mutation not detected in either parent; sporadic*: *RPS19* mutation not detected in either parent, but father has isolated raised eADA activity; familial: RPS19 mutation detected in other affected relatives.
†Previously reported in Draptchinskaia *et al* (1999).
‡Previously reported in Willig *et al* (1999).

RPS19 sequence analysis

Prevalence of RPS19 mutations. Fifteen different mutations were detected in 16 probands from a total of 104 families (15%), detailed in Table I. Four families have been included in previous reports (Draptchinskaia *et al*, 1999; Willig *et al*, 1999); 12 are reported here for the first time, including two patients found to have intragenic loss of heterozygosity (LOH), as described below. All mutations were heterozygous, affecting a single allele only. The prevalence of RPS19 mutations compares with published reports of four of 20 families (20%) (Cmejla *et al*, 2000) and four of 24 families (17%) (Matsson *et al*, 1999). A large collaborative European study reported *RPS19* mutations in 38 of 172 new families (22%) (Willig *et al*, 1999). An Italian study showed 21% of DBA patients to have *RPS19* mutations, but this figure included more than one affected individual from the same family (Ramenghi *et al*, 2000).

Polymorphisms. Willig *et al* (1999) reported two linked polymorphic sequence variations affecting intron 2. This was confirmed by Proust *et al* (2003), who also described an additional single nucleotide polymorphism (SNP) at position +14 of intron 4 (A > G), which was tightly linked to the intron 2 polymorphisms. In this study, we found a further two SNPs; a previously unreported C > T at position +395 of intron 4, and C > T at +38 of intron 1, which probably represents a previously described polymorphism (Willig *et al*, 1999). We found that these two SNPs were also tightly linked with the other introns 2 and 4 polymorphisms, generating just two haplotypes, as shown in Table II. The intron 1 polymorphism could only be determined in 65 patients, being close to the end of a PCR fragment, but followed the predicted pattern in each case. Like Proust *et al* (2003), we could find no evidence for a functional effect; the haplotypes occur in approximately equal proportions in probands, as in

Table II. Tight linkage of polymorphic sequence variations in introns 1, 2 and 4 results in two major *RPS19* haplotypes, equally prevalent in probands and normals (Proust *et al*, 2003), with loss of the paternal allele (nt: not tested).

	Intron 1	Intron 2		Intron 4	
	+38	+79	+89	+14	+395
Haplotype 1	AGG**C**GG	GGT**C**CCTGGCAGG**C**G		AG**A**GG	TT**T**TT
Haplotype 2	AGG**T**GG	GGT - CCTGGCAGG**G**G		AG**G**GG	TT**C**TT

normals (Proust *et al*, 2003), and there was no difference between subgroups of patients with respect to steroid responsiveness, presence of absence of physical anomalies, or age at presentation (data not shown).

Other sequence variations in non-coding regions. A single nucleotide difference G > A in the 5′ non-coding region was found in four probands at 1047 bp upstream from the initiation codon. None had any other detectable *RPS19* sequence changes. In one family, heterozygosity for G > A was shared by the mildly anaemic mother and transfusion-dependent proband, but not by two unaffected siblings. However, in a second family with an autosomal dominant pattern of inheritance, this nucleotide change did not co-segregate with DBA. In two further cases, parental samples were not available to confirm whether or not this represented an inherited SNP. There was no linkage with the common haplotypes, both probands with sporadic DBA being homozygous for haplotype 1, despite being heterozygous for G > A. Even if this upstream SNP does represent a polymorphic variation, it was not reported in 100 control chromosomes from 50 normal donors (Willig *et al*, 1999), which suggests that it is disproportionately represented in DBA, and thus that it may have functional significance. It is included within the upstream sequence conserved between mouse and man identified by Da Costa *et al* (2003a), but not within the region associated with promoter activity in their study in fibroblasts (Da Costa *et al*, 2003a). However, it might still influence erythroid specific expression. Interestingly, the G > A change generates putative binding sites for the Wilms tumour suppressor WT1 gene product (Nakagama *et al*, 1995) and for nuclear factor E2F (Thalmeier *et al*, 1989).

A further, previously unreported variation in the non-coding sequence (G > C at position +18 of intron 4) was observed in one patient, but not in her affected half-brother or their father in a family with DBA previously shown not to co-segregate with 19q (Gustavsson *et al*, 1998). No maternal sample was available to confirm that this represents a rare inherited SNP.

In this study, a 4 bp insertion at position −633 in the 5′ non-coding region found in one patient was categorized as a mutation. It has not been observed in any normal chromosomes (Willig *et al*, 1999) and resembles insertion/deletion mutations at a similar position in published studies (Willig *et al*, 1999; Ramenghi *et al*, 2000) (Table III). A MatInspector search for changes in potential regulatory motifs (Quandt *et al*, 1995) revealed the gain of a putative site for H6 homeodomain MX3/nkx5.1 (Amendt *et al*, 1999), and the disruption of a putative binding site for heat shock factor 1 (Kroeger & Morimoto, 1994), as a result of the 4 bp insertion at −633 reported in this study, but not the −629 to −625 deletion (Willig *et al*, 1999). A 4 bp insertion at −629/−625 was reported to have no effect on *RPS19* promoter function in a transient expression assay in a human fibroblast cell line (Da Costa *et al*, 2003a).

Intragenic LOH. Two patients showed a mixed pattern of heterozygosity and apparent homozygosity for the common polymorphisms described above. Haplotype analysis of the parents in each family demonstrated that the apparent breakdown in the usually tight linkage was the result of intragenic LOH. There was loss of the paternal allele in both cases, affecting the introns 1 and 2 polymorphisms in one case, and the intron 4 SNPs in the other (Table IV). These results were verified by repeat sequencing in both orientations of the freshly amplified product for all family members. While the endpoints of the deletions have not been mapped, these results are consistent with an intragenic rearrangement/deletion with a breakpoint in intron 3, which was also the site of the 19q breakpoint in the index case with t(X;19) and *RPS19* rearrangement (Draptchinskaia *et al*, 1999).

Parental origin of de novo RPS19 mutations. In two patients with point mutations, the proband was heterozygous for the common haplotypes, while one or both parents were homozygous, thus providing a means to demonstrate the parental chromosome of origin of the mutation by sequencing individual cloned alleles. In proband E63, a missense mutation (167G > A) was shown to be *in cis* with haplotype 2, which was paternal in origin, the proband's mother being homozygous for haplotype 1. The 34C > T mutation in patient E49 was similarly shown to have arisen on the paternal allele, in this case on haplotype 1. Although the mutations thus arose on the paternal allele in all four patients in this study for whom the parental chromosome of origin could be identified, one patient reported in the literature had a deletion affecting the maternal allele (Gustavsson *et al*, 1998). In other patients with *RPS19* mutations in our study, the polymorphisms were non-informative, either because the proband was not heterozygous, or the mutation was on an allele with a haplotype shared by both parents.

Discussion

We have undertaken a detailed analysis of DBA patients and their first-degree relatives in a population-based study to clarify and extend previous reports of raised eADA activity in parents or siblings of some children with apparently sporadic DBA (Glader *et al*, 1983; Whitehouse *et al*, 1986; Filanovskaia *et al*, 1993; Willig *et al*, 1998). In 85% of the families included in this study, the proband was the first family member in whom a diagnosis of DBA had been considered. However, haematological abnormalities and/or raised eADA activity could be detected in 31% of families with previously unsuspected familial DBA. Thus, in only 58% of probands could the DBA be categorized as being *de novo*. This figure may still represent an overestimate of sporadic DBA, as our results clearly demonstrate that normal parental results do not preclude the possibility of a subsequent baby also having DBA. In 12 of 60 families in this study (20%), there was more than one affected child despite both parents being

Table III. Details of *RPS19* mutations detected in 16 probands from a total of 104 families.

Ref.	Patient	Sex	Age*	Anomalies	Growth ret†	SR	Status‡	Mutation§	Effect	Comments
(A) Nonsense mutations										
UK¶	E71	f	2 m	Facial	y	y	SDep	280 C → T	Arg94Stop	
a	I	m	4 m	None	nk	n	TD	280 C → T	Arg94Stop	
b	G7	f	2 m	None	y	y	SDep	280 C → T	Arg94Stop	Familial
c	12	f	2 m	None	nk	n	nk	280 C → T	Arg94Stop	Familial – mother silent phenotype
b	I8	f	2 m	None	n	n	TD	280 C → T	Arg94Stop	Familial
b	I9	f	Birth	Glaucoma	n	y	TD	280 C → T	Arg94Stop	Familial
b	G27	m	nk	nk	nk	nk	nk	280 C → T	Arg94Stop	
UK¶	E49	f	3 m	None	n	n	TD	34 C → T	Gln12stop	
b	F23	f	1 m	Renal, facial	y	n	dec'd	34 C → T	Gln12stop	
b	I7	m	Birth	None	n	n	TD	31 C → T	Gln11stop	
a	C	f	1 m	None	nk	n	TD	31 C → T	Gln11stop	
(B) Missense mutations										
UK¶	E98	f	6 w	None	y	y	SDep	3 G → T	Met1Ile	
a	K	f	1 m	None	nk	n	TD	3 G → A	Met1Ile	
c	1	m	2 m	None	nk	n	nk	3 G → C	Met1Ile	
c	17	m	Infancy	None	nk	n	nk	3 G → T	Met1Ile	
b	F9	m	Birth	None	nk	y	nk	1 A → G	Met1Val	Familial
UK¶	E63	m	nk	None	y	n	TD	167 G → A	Arg56Gln	
b	F32	f	1 m	None	n	pr	TD	167 G → A	Arg56Gln	
b	F28	m	1 m	None	nk	pr	TD	167 G → A	Arg56Gln	
b	F7	m	3 m	None	nk	n	TD	167 G → A	Arg56Gln	
d	CZ 7	f	1 m	None	n	nk	TD	167 G → A	Arg56Gln	Familial (sister of CZ6)
d	CZ 6	f	2 m	None	y	nk	Remission	167 G → A	Arg56Gln	Familial (sister of CZ7)
UK¶	E85	m	2 m	none	n	n	TD/GCSF	169 G → C	Ala57Pro	Neutropenia
UK¶	E28	f	8 m	None	n	nt	None	182C → A	Ala61Ser	
(C) Insertion/deletion mutations in coding regions										
UK¶	E13	m	4 m	Facial, toe	n	y	SDep	250/251 del AG	fs 84/no stop	
c	11	m	1 m	None	nk	y	nk	237 ins G	fs 81/no stop	
b	I4	m	1 m	None	nk	y	Remission	238/239 ins G	fs 81/no stop	
UK¶	E83	m	10 m	Face, thumb	nk	n	TD	331 del C	fs 110/stop 110	
e	T	f	1 yr	None	nk	nk	TD	309 del G	fs 103/stop 110	Osteosarcoma
UK¶	61	m	1 m	Toe	y	n	TD	344/345 ins AA	fs 116/stop 124	
b	I2	m	1 m	None	nk	n	TD	341 del A	fs 115/stop 123	
UK¶	73	f	3 m	None	n	y	None	383/4 del AA	fs 130/no stop	
UK¶	10	m	3 m	None	n	y	SDep	383/4 del AA	fs 130/no stop	Familial, variable phenotype
c	3	f	Birth	None	nk	n	nk	384/5 del AA	fs 130/no stop	
b	F64	f	11 m	Microcephaly	y	y	Remission	390/391del 2nt	fs 130/no stop	
d	CZ 16	m	3 w	None	y	nk	TD	nt 386 ins 8	fs 131/no stop	
(D) Promoter mutations										
UK¶	E54	m	6 w	None	n	n	TD	ins GCCA −633		
c	15	m	6 m	None	nk	pr		ins AGCC −633		Familial – mother silent phenotype
b	F21	m	2 m	None	y	n	TD	del 4nt (−629/−625)		Additional *RPS19* mutation
b	F25	f	1 m	None	y	y	SDep	del 4nt (−629/−625)		Additional *RPS19* mutation
b	F1	m	Birth	None	nk	n	TD	del 4nt (−629/−625)		Familial, variable phenotype
(E) Mutations affecting splice sites										
UK¶	E3	m	13 m	None	n	n	None	agATG → atATG	IVS1 acceptor	Familial, mild phenotype

Table III. *continued*

Ref.	Patient	Sex	Age*	Anomalies	Growth ret†	SR	Status‡	Mutation§	Effect	Comments
UK¶	E64	m	34/40	None	y	n	TD	AAgt → Aaat	IVS2 donor	
b	F66	f	2 m	None	nk	y	SDep	del (+3 to +6) IVS2	IVS2 donor	Familial variable phenotype
(F) Large deletions										
UK¶	75	m	Birth	Face, thumb, renal	nk	n	TD	LOH 3′ to exon 3		
UK¶	25	f	2 m	Face, learning	y	pr	TD	LOH 5′ to exon 4		
b,f	S10	f	1 m	Renal, branchial cyst	y	pr	TD	t(X;19) original	Breakpoint IVS3	
b,g,h	S11	m	2 m	Skeletal, learning	nk	pr	TD	Total allele deletion		
i	MH	m	4 m	Facial, learning, skeletal	y	y	Remission	19q 3 Mb deletion	Includes RPS19	
b,h	I24	f	2 m	Learning, skeletal	y	y	TD	t(8;19) large deletion		

w, weeks; m, months; yr, year; SR, steroid responsive; y, yes; n, no; pr, partial response; nk, not known; del, deletion; ins, insertion; LOH, loss of heterozygosity; fs, frameshift; IVS, intervening sequence; dec'd, died; TD, transfusion dependent; GCSF, granulocyte colony stimulating factor; SDep, steroid dependent.

References: a: Proust *et al* (2003); b: Willig *et al* (1999); c: Ramenghi *et al* (2000); d: Cmejla *et al* (2000); e: Matsson *et al* (1999); f: Gustavsson *et al* (1997a); g: Tentler *et al* (2000); h: Gustavsson *et al* (1998); i: Cario *et al* (1999).

*Age: age at presentation;

†Growth ret: growth retardation defined by height <3rd centile for age.

‡Status: haematological status at time of study; remission: transfusion and steroid independent (Hb and MCV not known).

§*RPS19* nomenclature: mRNA numbering from initiation codon for coding sequence mutations; mutations in 5′ non-coding region: nucleotides 5′ to initiation codon in genomic sequence.

¶UK: proband from this study.

Table IV. Pattern of *RPS19* polymorphisms in two probands and their first-degree relatives.

	Intron 1 +38	Intron 2 +79	Intron 2 +89	Intron 4 +14	Intron 4 +395
E75	C/T	CC/CCC	C/G	G	C
Father	nt	CCC	C	A	T
Mother	nt	CC/CCC	C/G	A/G	T/C
Brother	nt	CC/CCC	C/G	A/G	T/C
E25	C	CCC	C	A/G	C/T
Father	nt	CC	G	G	C
Mother	nt	CCC	C	A	T
Sister	nt	CC/CCC	C/G	A/G	T/C

nt, not tested.

The apparent breakdown in tight linkage of *RPS19* polymorphisms is explained in each case by intragenic hemizygosity.

haematologically normal, including having normal eADA activity. In five of these families, two children had overt DBA, while in seven there was relatively mild anaemia or isolated raised eADA in one or more siblings. The significance of high eADA activity, even in the absence of other haematological abnormalities, has been endorsed by the report of a sibling who presented with overt steroid-responsive red cell aplasia at puberty, having previously been found to have isolated raised eADA activity with normal haemoglobin and MCV (S. Howell, S. Ball, K. Orfali, A. Will, T. Carr, R. Stevens & R. Wynn, unpublished observations).

Our findings have important implications for genetic counselling, especially in the absence of a detectable *RPS19* mutation. In keeping with other series (Willig *et al*, 1999; Ramenghi *et al*, 2000) we found that it was not possible to distinguish between patients with and without *RPS19* mutations on the basis of clinical phenotype. Notably, the phenotype of an isolated increase in eADA activity was also seen in families without *RPS19* mutations, despite an earlier report from the French registry that raised eADA activity co-segregated with 19q13 (Willig *et al*, 1998), in a study that predated the identification of *RPS19* as the first gene responsible for the previously reported 19q linkage (Gustavsson *et al*, 1997b; Draptchinskaia *et al*, 1999). Caution must be applied in genetic counselling, even for families in which the proband has an apparently *de novo RPS19* mutation, as shown by the report of two sisters in the Czech registry who had identical *RPS19* mutations, which could not be detected in either parent (Cmejla *et al*, 2000). Germline mosaicism affecting one parent provides the likely explanation for this observation. The variable pattern of inheritance further complicates genetic counselling. While the occurrence of more than one affected sibling with phenotypically normal parents is usually assumed to reflect recessive inheritance (Gustavsson *et al*, 1998; Ramenghi *et al*, 1999), all reported *RPS19* mutations to date have been heterozygous, with one normal *RPS19* allele, even in two families with consanguinity (Proust *et al*, 2003). Autosomal dominant inheritance with incomplete penetrance provides the probable alternative explanation.

Genetic counselling in DBA is further complicated by the wide range of severity, with inter- and intrafamilial phenotypic variability, even between individuals with identical *RPS19* mutations. In this regard, DBA resembles the more familiar

pattern of β-thalassaemia intermedia, with a clinical spectrum that ranges from silent or mild to severe transfusion-dependent anaemia. In the example of β-thalassaemia, the clinical phenotype is clearly influenced by the nature of the primary β-globin mutation (Weatherall, 2000, 2001). We therefore undertook a literature search for patients with identical mutations to those found in our study, or with mutations predicted to have an equivalent effect to determine whether any genotype: phenotype correlation could be established (Table III). Some individuals with mutations had been reported in more than one published series, and care was taken to ensure that duplicate results were not included.

This approach provided evidence that the nature of the *RPS19* mutation can influence the clinical phenotype. This was predictably most apparent with large deletions or rearrangements, which were associated with multiple non-haematological abnormalities, including learning difficulties, which is not a common feature of DBA (Ball *et al*, 1996). While this finding would be consistent with a contiguous gene syndrome (Gustavsson *et al*, 1998; Cario *et al*, 1999; Tentler *et al*, 2000), there was also an unequal distribution of physical anomalies, not including growth retardation, across the other broad categories of mutations observed in this study. In contrast to all of the six patients with large deletions (100%), no physical anomalies were reported in any of the eight patients with splice site or promoter region mutations (0%), and in only three of 31 probands with missense or nonsense point mutations (10%), and in four of 12 with coding region insertion/deletion frameshift mutations (33%).

There was also a possible genotype:phenotype correlation with respect to haematological severity, as shown by the mutations found in probands and families with a mild or variable haematological phenotype. This 'DBA intermedia' phenotype was apparent in two of three families with splice site mutations, and also in two of the three families in which insertions or deletions in the promoter region were the only *RPS19* mutations detected. A missense mutation (Ala61Ser) affecting a non-highly conserved residue (Willig *et al*, 1999) was detected in a child with only mild anaemia, requiring no treatment. These might therefore be considered to be '*RPS19*$^{+}$' mutations, by analogy with the β-globin mutations associated with β-thalassaemia intermedia (Weatherall, 2000, 2001). By contrast, a recurring missense mutation, Arg56Gln, which affects a highly conserved residue (Willig *et al*, 1999), was more likely to be associated with sporadic, more typical transfusion- or steroid-dependent anaemia ('DBA major'), and thus might represent an '*RPS19*$^{-}$' mutation. However, mutations affecting similarly highly conserved arginine residues at positions 62 and 101 have been associated with a variable phenotype in the literature (Draptchinskaia *et al*, 1999; Willig *et al*, 1999).

Da Costa and colleagues have studied the subcellular localization and protein expression of other mutated RPS19 genes in Cos-7 cells. The missense point mutations Val15Phe and Gly127Gln were found to disrupt a nucleolar localization signal, and to lead to reduced protein levels (Da Costa *et al*, 2003b). C-terminal truncations showed similar results, in effect causing a quantifiable defect in protein expression. By contrast, missense mutations affecting the highly conserved residues 52–62 did not affect nucleolar localization, and were associated with normal protein levels (Cretien *et al*, 2003). The further elucidation of the functional effect of such missense mutations should help to clarify the structure:function relationship of *RPS19* in DBA. However, it is still not yet clear whether *RPS19* mutations cause DBA by an effect on ribosomal function, whether by a global effect on translation, or by a more selective influence on the translation of a critical protein (Mauro & Edelman, 2002). There is increasing evidence that ribosomal proteins may also have extra-ribosomal functions (Zimmermann, 2003). Cytoplasmic ribosomal protein S19 has been shown to interact with fibroblast growth factor 2 in murine fibroblast cell lines (Soulet *et al*, 2001), and ribosomal protein S19 homodimers have monocyte chemotactic activity in rheumatoid synovium (Nishiura *et al*, 1996). The residues important for ribosomal protein S19 homodimerization have been shown to be Lys122 and Gln137 (Nishiura *et al*, 1999), neither of which has yet been identified as a site of missense point mutations in DBA.

It is clear that other factors must influence the final phenotype of DBA in addition to the causal *RPS19* mutation (Wolf, 1997; Weatherall, 2001). Factors that modify the level of expression of *RPS19* may be important, analogous to the modulation of the clinical phenotype by a polymorphism affecting the level of expression of the wild-type ferrochelatase gene in erythropoietic protoporphyria, which is also characterized by autosomal dominant inheritance with incomplete penetrance (Gouya *et al*, 2002). The future identification of the gene responsible for linkage to 8p (Gazda *et al*, 2001) will be an important step, and studies of complex pedigrees with variable phenotype should help to elucidate the role of *RPS19* mutations in the, as yet, enigmatic pathogenesis of DBA.

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