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ORIGINAL INVESTIGATION

Clinical and molecular genetic features of ARC syndrome

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Abstract Arthrogryposis, renal dysfunction and cholestasis (ARC) syndrome (MIM 208085) is an autosomal recessive multisystem disorder that may be associated with germline *VPS33B* mutations. VPS33B is involved in regulation of vesicular membrane fusion by interacting with SNARE proteins, and evidence of abnormal polarised membrane protein trafficking has been reported in ARC patients. We characterised clinical and molecular features of ARC syndrome in order to identify potential genotype-phenotype corre-

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lations. The clinical phenotype of 62 ARC syndrome patients was analysed. In addition to classical features described previously, all patients had severe failure to thrive, which was not adequately explained by the degree of liver disease and 10% had structural cardiac defects. Almost half of the patients who underwent diagnostic organ biopsy (7/16) developed life-threatening haemorrhage. We found that most patients (9/11) who suffered severe haemorrhage (7 post biopsy and 4 spontaneous) had normal platelet count and morphology. Germline *VPS33B* mutations were detected in 28/35 families (48/62 individuals) with ARC syndrome. Several mutations were restricted to specific ethnic groups. Thus p.Arg438X mutation was common in the UK Pakistani families and haplotyping was consistent with a founder mutation with the most recent common ancestor 900–1,000 years ago. Heterozygosity was found in the *VPS33B* locus in some cases of ARC providing the first evidence of a possible second ARC syndrome gene. In conclusion we state that molecular diagnosis is possible for most children in whom ARC syndrome is suspected and *VPS33B* mutation analysis should replace organ biopsy as a first line diagnostic test for ARC syndrome.

Introduction

Arthrogryposis, renal dysfunction and cholestasis (ARC) syndrome (OMIM 208085) typically presents with neonatal cholestatic jaundice, renal tubular leak and hypotonia-related arthrogryposis. Other features variably reported include ichthyosis, mild dysmorphic signs, absent corpus callosum and recurrent infections resulting in severe metabolic acidosis, worsening nephrogenic diabetes insipidus and rarely liver failure. Significantly, a platelet storage pool defect similar to grey platelet syndrome is reported to occur in ~ 25% of cases (Eastham et al. 2001). Previously, we mapped the ARC disease locus to 15q26.1 and identified 9 different germline *VPS33B* mutations in 14 of 15 kindred tested (Gissen et al. 2004). In order to further define the molecular pathology of ARC syndrome we undertook molecular genetic studies in a further 20 ARC syndrome kindreds and obtained detailed clinical information on 62 affected individuals from at least 14 different ethnic backgrounds providing the largest cohort of ARC patients yet analysed.

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Patients and methods

Patients

Clinical data was recorded prospectively for living patients and information on deceased patients was obtained from the case notes and pathology records. Eleven of the 35 families in the current report have also been reported previously (Table 1). A clinical diagnosis of ARC syndrome was based on a triad of arthrogryposis, renal tubular dysfunction and cholestasis with a low γ-glutamyl transpeptidase (gGT) activity. Clinical details were available for 62 patients (35 males) of varied ethnic background (Table 1). Study protocols were approved by the South Birmingham research ethics committee.

Genotyping

DNA was extracted from blood by standard methods. To estimate the age of the most recent common ancestor for the p.Arg438X mutation we analysed DNA from eight affected individuals from eight unrelated consanguineous families from Mirpur region of Northern Pakistan. The extent of the common haplotype in the ARC locus was determined by genotyping fluorescently labelled microsatellite markers, which were identified using public genome databases and deCODE, Marshfield and Généthon genetic maps. Novel microsatellite markers from the ARC locus were identified using the current chromosome 15 draft genome sequence (NT_033276 and NT_033277). Patients' DNA was amplified by PCR with novel primers flanking microsatellite markers as described previously (Gissen et al. 2004). PCR products were run on an ABI 3730 DNA analyser and then analysed with genemapper software package (ABI). In order to estimate population frequency of the individual marker alleles we genotyped DNA from 30 anonymised ethnically matched controls (UK Asians).

Table 1 Clinical features in ARC patients

Pedigree, no. of affected, consang, ethnicity	Renal features	Hepatic features	CNS/ skeletal abnormalities	Antenatal problems, gi, skin, endocrine	Haematology, immunity	Dysmorphic features, cardiac defects, other	Survival, previously reported-references
1) 7, yes, Pakistani	RFS 6/6, NDI 6/6, TRP reduced in 2/2. USS loss of corticomedullary differentiation (2/2). RBx: tubular atrophy, nephrocalcinosis (1/1)	NC in 6/6. transaminases normal/mildly raised, Alk Phos 2–3× normal, Bn 40–150 mmol/l (90% conjugated) in 7/7 TBIDA non-excreting in 2/2 LBx in 2: giant-cell hepatitis and lipofuscin deposition (2/2), biliary hyplasia (2/2)	AMC in 7/7, hypotonia in 7/7, CT scan: absent corpus callosum in 1/4	BW < 10% and FTT in 6/6l, enteral supplementation in 4/7. Diarrhoea in 4/7 Oligohydramnios noted in 3. Ichthyosis (7/7)	GPS in 1/6, severe bleeding after organ biopsy in 2/2. Recurrent infections (7/7)	Low set ears, proximally inserted thumbs and big toes, sloping forehead (3/7). Persistent foramen ovale in 1/4	3 weeks–4 months Eastham et al. (2001) for 2/7
2) 7, yes, Pakistani	RFS (7/7). NDI (7/7), reduced TRP (1/1), raised PTH and protein/creatinine ratio (658) 1/1, raised to normal calcium, low phosphate in 4/4, low albumin (20 g/L in 1/7) USS in 2: small hyperechoic kidneys, loss of cortico-medullary differentiation 2/2 RBx in 1: tubular microcalcification and glomerular immaturity	NC in 7/7, normal-mildly raised transaminases, Bn 150–200 mmol/L (90% conjugated) in 7/7, Alk Phos 2,000–4,000 IU/L in 7/7 (predominantly bone isoform in 1/1 studied) TBIDA scan non-excreting in 4/4. LBx in 4: giant-cell hepatitis 4/4, lipofuscin deposition 2/4	AMC in 5/7, hypotonia in 7/7. Normal cerebral USS (3/3)	BW < 10% and FTT in all. Enteral feeds in all, diarrhoea in 5/7. Ichthyosis in 7/7	Normal platelet count and morphology in all. Severe bleeding after organ biopsy in 2/4. Recurrent infections (7/7)	Low set ears, small chin, proximally inserted thumbs in 4/5. Secundum ASD in 1/4, hirsutism in 3/4	1 week–7 months Horslen et al. (1994) for 2/7
3) 3, yes, Pakistani	RFS, NDI in 3/3	NC, normal transaminases and Alk Phos 3,000 IU/L in 3/3	AMC in 3/3	BW < 10% and FTT in 2 Diarrhoea in 2. Ichthyosis in 2	Severe haemolytic anaemia in 2/2, GPS in 1/2	PFO in 1/2	2–4 weeks
4) 2, yes, Pakistani	RFS, NAG/creatinine ratio > 2,375 U/mmol (normal 3.5–27.3). Raised calcium/creatinine ratio 2.2 (normal up to 0.7), TRP 54% (normal 80–100%), albumin/creatinine ratio 0.79 (normal up to 0.1). All in 1/1 USS normal in 1/1	NC, normal ALT, AST, gGT, Alk Phos, Bn 71 mmol/L (90% conjugated) (1/1) TBIDA non-excreting in 1/1 LBx in 1/1: paucity of bile ducts and bile ducts are small. Lobules show giant cell transformation of hepatocytes, cholestasis, activated Kupffer cells	AMC, multiple fractures, slender long bones and ribs on skeletal X-ray (1/1) MRI brain normal (1/1)	BW < 10%, FTT, diarrhoea. hirsutism, ichthyosis, lax skin (1/1). Second trimester miscarriage with renal abnormalities on pm (homozygous for mutation in *VPS33B*)	Severe bleeding after LBx in 1/1. Normal FBC and platelet morphology, recurrent infections (1/1)	Microcephaly, proximimally inserted thumbs, beaked nose (1/1)	5 months Eastham et al. (2001) for 1/2

Table 1 continued

Pedigree, no. of affected, consang, ethnicity	Renal features	Hepatic features	CNS/ skeletal abnormalities	Antenatal problems, gi, skin, endocrine	Haematology, immunity	Dysmorphic features, cardiac defects, other	Survival, previously reported-references
5) 2, yes, Pakistani	RFS, NDI in 2/2	NC: Bn 100 (90% conjugated), normal ALT, AST, gGT in 2/2 TBIDA non-excreting in 1/1 LBx: giant cell hepatitis and cholestasis in 1/1	AMC, hypotonia in 2/2	BW < 10%, FTT in 2/2 Oligohydramnios in 2. Ichhthyosis and lax skin in 2	Normal platelet count and morphology in 2/2	Sloping forehead in 2/2, low set ears in 2/2	5 days and 3 months Eastham et al. (2001)
6) 2, yes, Pakistani	RFS, NDI in 1/1 USS: both kidneys contained multiple small echogenic foci not casting an acoustic shadow in 1/1	NC in 2/2, Bn 180 mmol/L, 90% conjugated, mildly raised ALT, AST, gGT normal, Alk Phos 2,872 IU/L in 1/1	AMC in 2/2	BW < 10%, FTT in 2/2. Third trimester miscarriage with renal dysplasia (unknown mutation status). Hirsutism, Ichthyosis, lax skin in 2/2	Normal platelet count and morphology. Prolonged bleeding time in 1/1	Low set ears in 2/2	6 and 7 months
7) 2, yes, Pakistani	RFS and NDI in 2/2	NC: Bn 45 (90% conjugated), normal ALT, AST, gGT in 1/1 TBIDA non-excreting in 1/1	AMC and long bone fractures at birth in 2/2	BW < 10% and FTT in 2 Hypothyroidism in 1/1. Lax skin folds in 1/2	Normal platelet count and morphology in 2/2		5 months Eastham et al. (2001) for 1/2
8) 1, yes, Pakistani	RFS, NDI, TRP 25% USS: reduced corticomedullary differentiation	NC, normal ALT, AST, gGT, Bn 90 mmol/L (90% conjugated)	AMC	FTT, hypothyroidism, ichthyosis. Lax skin folds	Normal platelet numbers and morphology, abnormal platelet aggregation		4 months
9) 1, yes, Pakistani	RFS, proteinuria, albumin 24 g/L	NC Bn 253 mmol/L (conjugated 80%), ALT, gGT normal, Alk Phos normal. TBIDA normal	AMC	BW < 10%, FTT, lax skin Transient hypothyroidism	Normal platelet count and clotting. Recurrent infections	Short neck, low set ears, large hands. Secundum ASD	4 months Eastham et al. (2001)
10) 1, yes, Pakistani	RFS	NC, conjugated hyperbilirubinaemia	AMC				NK
11) 1, yes, Pakistani	RFS	NC, conjugated hyperbilirubinaemia	AMC				NK
12) 1, yes, Pakistani	RFS	Conjugated hyperbilirubinaemia	AMC				NK

Table 1 continued

Pedigree, no. of affected, consang, ethnicity	Renal features	Hepatic features	CNS/ skeletal abnormalities	Antenatal problems, gi, skin, endocrine	Haematology, immunity	Dysmorphic features, cardiac defects, other	Survival, previously reported-references
13) 1, yes, Pakistani	RFS, NDI, creatinine 60–244, hypernatraemiac dehydration, albumin < 20 USS generally hyperechoic, loss of corticomedullary differentiation	Bn 260 (90% conjugated), normal ALT, AST, gGT, Alk Phos	AMC	BW = 25%, FTT, hirsutism and ichthyosis	Haemolytic anaemia, abnormal clotting APTT 80, TT 16.8, fibrinogen 1.2, sepsis. Pulmonary haemorrhage		6 weeks
14) 1, no, Scottish	RFS, NDI.	NC, ALT, AST, gGT normal, Bn 100 mmol/L (80% conjugated).	AMC, hypotonia.	BW = 25%, FTT. Lax skin, ichthyosis	FBC normal	Arachnodactyly	5 months
15) 1, no, Phillipino	RFS, USS normal	Mildly raised ALT, AST, normal gGT, conjugated hyperbilirubinaemia	AMC, severe truncal hypotonia, Abnormal nerve conduction Dysgenesis of corpus callosum	FTT. Severe ichthyosis	FBC normal		7 months
16) 3, no, Portuguese	RFS	Conjugated jaundice in 3/3 TBIDA non-excreting in 1/1	AMC in 3/3, normal CT scan in 1/1	BW < 10%, FTT, lax skin, ichthyosis in 3/3. Nuchal translucency increased on antenatal scan, oligohydramnios in 1/1	FBC normal (3/3), GI haemorrhage recurrent 1/3		3, 5 and 6 months
17) 1, yes, Turkish	RFS, raised PTH	Conjugated hyperbilirubinaemia, normal ALT, AST, gGT, Alk Phos 1,500 mmol/L TBIDA non-excreting	AMC Hypoplastic corpus callosum	Exocrine pancreatic insufficiency, parenteral nutrition	FBC normal		7 months Denecke et al. (2000)
18) 1, no, Italian	RFS, USS: poor cortico-medullary differentiation	Conjugated hyperbilirubinaemia, mildly raised ALT, AST, normal gGT	AMC, hypotonia, Brain MRI: increased T1 weighted signal in basal ganglia, capsular, corticospinal and rolandic area bilaterally, hypoplastic corpus callosum	FTT, no diarrhoea, on enteral nutrition	Raised APTT 52 (normal 35), died after first infection-pneumonia		14 months
19) 2, yes, Italian	RFS, NDI in 2/2 RBx showed nephrocalcinosis, renal tubular cell degeneration in 1/1	Conjugated hyperbilirubinaemia, normal ALT, AST, gGT in 2/2 LBx: lipofuscin deposition in 1/1	AMC in 2/2, anterior horn cell depletion in 1/1	FTT, ichthyosis in 2/2		Infant deaths in the family	2 months Di Rocco et al. (1995)

Table 1 continued

Pedigree, no. of affected, consang, ethnicity	Renal features	Hepatic features	CNS/ skeletal abnormalities	Antenatal problems, gi, skin, endocrine	Haematology, immunity	Dysmorphic features, cardiac defects, other	Survival, previously reported-references
20) 1, no, Italian	RFS, NDI	Conjugated hyperbilirubinaemia	AMC	FTT	Normal FBC, recurrent sepsis		3 months Di Rocco et al. (1995)
21) 2, no, Portuguese	RFS, NDI in 1/1	NC, slightly raised ALT, AST, normal gGT. Alk Phos 2,500 mmol/L in 1/1 TBIDA non-excreting in 1/1 LBx: intracellular and canalicular cholestasis, giant cell hepatitis in 1/1	AMC, hypotonia, abnormal nerve conduction and EMG. Neurogenic muscle atrophy. MRI showed poor grey/white matter differentiation, thin corpus callosum. All in 1/1	BW < 10%, feed intolerance, severe GOR (dilated esophagus with poor motility), FTT in 1/1	GPS, absent alpha granules on EM in 2/2, intra-abdominal bleeding post LBx in 1/1 Recurrent infections in 1/1. Second trimester termination of mutation-positive fetus		2 months Hayes et al. (2004)
22) 1, no, French	RFS USS: multilobulated kidneys with cortical hypoechogenicity and poor cortico-medullary differentiation	NC, normal gGT, ALT, AST LBx: intrahepatic cholestasis and intralobular inflammation	AMC, hypotonia, amyotrophy of the limbs	FTT	Increased bleeding time, normal platelet number and size, but decreased platelet granularity. Bleeding after organ biopsy		2 months
23) 2, yes, Arab (Isr)	RFS, NDI in 2/2	NC LBx in 1/1: paucity of intrahepatic bile ducts	AMC in 2/2	FTT and diarrhoea in 2/2			4 months
24) 3, yes, Arab (Isr)	RFS, NDI in 3/3	NC in 3/3 LBx in 1/1: paucity of intrahepatic bile ducts	AMC in 3/3, neurogenic atrophy on muscle biopsy in 1/1	FTT, diarrhoea in 3/3			1–4 months
25) 1, no, Swiss	RFS	NC	AMC	FTT			6 months
26) 1, yes, Turkish	RFS	NC, mild elevation of ALT, AST, normal gGT	AMC	FTT			7 months
27) 1, yes, Arab (Saudi)	RFS, NDI	NC	AMC, hypoplastic corpus callosum, sensorineural deafness	FTT		VSD	6 months Abdullah et al. (2000)

Table 1 continued

Pedigree, no. of affected, consang, ethnicity	Renal features	Hepatic features	CNS/ skeletal abnormalities	Antenatal problems, gi, skin, endocrine	Haematology, immunity	Dysmorphic features, cardiac defects, other	Survival, previously reported-references
28) 1, no, Swedish	RFS, NDI	NC, normal transaminases	AMC, hypotonia, hypoplastic corpus callosum, deafness	FTT	Normal FBC and platelet morphology Died of pulmonary haemorrhage		20 months
29) 3, yes, Turkish	RFS in 3/3	NC, conjugated hyperbilirubinaemia in 3/3 TBIDA scan non-excreting in 2/2 LBx in 2/2 at age 6 weeks: some giant cells in 1/2, periportal fibrosis in 1/2, hepatocellular cholestasis in 2/2, bile duct hypoplasia in 2/2	AMC in 3/3	Birth weight < 10% in 3/3, FTT in 3/3, despite nutritiomal supplements. Partial parenteral nutrition in 2/3. Ichthyosis in 3/3 Raised lipoprotein (X30 upper limit of normal) and bile acids (X10 upper limit of normal) in 1/1 Oligohydramnios in 3/3	Normal full blood count and morphology in 3/3 Spontaneous gastrointestinal bleeding in 1/3	Recurrent infections in 3/3	4, 9 and 10 months
30) 1, yes, Arab (Saudi)	RFS, NDI, Mg/Creat 0.33 (normal < 0.09), TRP 6%, NAG/Creat 1,152 U/mmol, urine RBP/creat 72,877 μg/mmol (normal < 50) USS normal	NC, fluctuating conjugated hyperbilirubinaemia, mildly raised ALT, normal gGT	AMC, hypotonia	FTT, lipase 627 (normal 13–95), normal amylase. Lax skin, ichthyosis. Oligohydramnios noted in third trimester of pregnancy	Platelet count normal, platelets normal size but poorly granulated. Normal clotting, collagen/epinephrine closure time > 300 s (normal 86–166 s). Collagen/ADP Closure time 119 s (normal 61–144)	Recurrent infections, bowing of tibia, proximally inserted thumbs, low-set ears, small mandible	7 months
31) 1, yes, Turkish	RFS, structural renal abnormalities	NC, normal-mildly raised ALT, AST, ALT, Bn 150 IU/L (70% conjugated). LBx: giant cells hepatitis	AMC, hypotonia. Microcephaly, MRI: reduced myelination, reduced intracerebral volume, EEG showed lobe differences and slow waves	FTT	Normal FBC	VSD, premature closure of frontal suture	5 months

Table 1 continued

Pedigree, no. of affected, consang, ethnicity	Renal features	Hepatic features	CNS/ skeletal abnormalities	Antenatal problems, gi, skin, endocrine	Haematology, immunity	Dysmorphic features, cardiac defects, other	Survival, previously reported-references
32) 1, no, French	RFS, NDI	NC, conjugated hyperbilirubinaemia. Normal transaminases	AMC Brain MRI normal, EMG: axonal damage Sensorineural deafbess	FTT, enteral nutrition	Normal FBC		8 months
33) 1, yes, Pakistani	RFS	NC, conjugated hyperbilirubinaemia	AMC, bilateral hip dysplasia	FTT, enteral nutrition			2 months
34) 1, no, Tahitian	RFS USS: bilateral lithiasis and reduced corticomedullary differentiation	NC, conjugated hyperbilirubinaemia, normal transaminases and Alk Phos. USS normal	AMC, congenital bilateral hip dislocation Axial hypotonia MRI: hypoplasia of the corpus callosum with an hypersignal T2	Birthweight: 3,130 g Weight at 6 months: 3,600 g Diarrhea Enteral nutrition during daytime, and nasogastric feeding at night Nutrition with elemental feeds Vitamins and supplements	Platelets: normal size but reduced granularity Abnormal platelet aggregation. Reccurrent febrile episodes with no sign of infection		Alive at 8 months
35) 1, no, North European/ native South American	RFS USS normal	NC, conjugated hyperbilirubinaemia USS normal	AMC with bilateral hip dislocation Intracranial USS normal Deafness	Birth weight < 10% FTT Breast feeds Vitamins and supplements	Anaemia requiring blood transfusions		7 months

ADP adenosine diphosphate, *Alk Phos* alkaline phosphatase, *ALT* alanine aminotransferase, *AMC* arthrogryposis multiplex congenita, *ASD* atrial septal defect, *AST* aspartate aminotransferase, *Bn* bilirubin, *BW* birth weight, *CNS* central nervous system, Consang consanguinity, *EM* electron microscopy, *EEG* electroencephalogram, *EMG* electromyogram, *FBC* full blood count, *FTT* failure to thrive, *gGT* γ glutamyl transpeptidase, *GI* gastrointestinal, *GOR* gastroesophageal reflux, *GPS* gray platelet syndrome, *LBx* liver biopsy, *Mg* magnesium, *MRI* magnetic resonance imaging scans, *NAG* *N*-acetyl-beta-*D*-glucosaminidase, *NC* neonatal cholestasis, *NDI* nephrogenic diabetes insipidus, *No.* number, *PFO* persistent foramen ovale, *pm* post mortem examination, *PTH* parathyroid hormone, *RBP* retinol binding protein, *RBx* renal biopsy, *Ref* reference, *RFS* renal fanconi syndrome, *TBIDA* technetium labelled methyl bromoiminodiacetic acid scans, *TRP* total resorption of phosphate, *USS* ultrasound scan, *VSD* ventricular septal defect

Table 2 *VPS33B* mutations in patients with ARC syndrome

Pedigree number	Ethnic origin	Nucleotide alterations	Alterations in coding sequence	Exon	Status	Survival
01	Pakistani	c.1593C>T	p.Arg532X	21	Hom	3 weeks–4 months
02, 03, 05, 06, 07 08, 09, 10, 11, 12, 13, 33	Pakistani	c.1311C>T	p.Arg438X	18	Hom in all	1 week–7 months
04	Pakistani	c.89T>C	p.Leu30Pro	1	Hom	5 months
16	Portuguese	c.1518C>T	p.Arg507X	20	Hom	3 months
14	Scottish	**c.319C>T**	**p.Arg97X**	5	Het	5 months
		c.403+1G>T		6+1	Het	
18*	Italian	**c.556_557delCT**	**p.Leu175fsX219**	8	Hom	14 months
20	Italian	c.177+1G>A		2+1	Hom	3 months
21	Portuguese	c.853A>G	p.Arg507X	12–2	Het	2 months
		c.1518C>T		20	Het	
22	French	**c.940–2G>A**		13–2	Het	2 months
		?c.240–13delTT		4–13	Het	
24	Arab (Israel)	c.403+1G>A		6+1	Hom	1–4 months
27	Arab (Saudi Arabia)	c.1406–2A>G		19–2	Hom	6 months
28	Swedish	**c.498+1G>A**		7+1	Het	20 months
29	Turkish	**c.1406–1G>C**		19–1	Hom	5–9 months
30	Arab (Saudi Arabia)	c.348delC	p.Ser116fsX136	5	Hom	7 months
32	French	**c.151C>T**	**p.Arg51X**	1	Het	8 months
		c.433_442delTTGCTGCCTC	**p.Leu145fsX151**	7	Het	
34	Tahiti	**c.1208delT**	**p.Leu403fsX414**		Het	Alive at 8 months
35	Northern European/native South American	**c.277C>T**	**p.Arg93X**	4	Het	7 months
		c.369_370delTG	**p.Cys123X**	6	Het	

Nucleotides are numbered from A of the initiation codon (ATG). *Het* heterozygous, *hom* homozygous, *fs* frameshift, *del* deletion, *X* stop. Novel mutations highlighted in *bold*

Mutation analysis

Mutation analysis was performed by direct sequencing of coding exons and flanking sequences (Gissen et al. 2004). All mutations were verified bidirectionally. Twenty distinct mutations (11 novel) were identified comprising 7 nonsense, 5 frameshift and 8 splice site mutations (Table 2). Segregation of the putative disease-causing mutations was analysed in the affected families. We used DNA from 100 anonymised controls to assess population frequencies of DNA variants. DNA from 100 anonymised healthy UK Asians was used as controls for the UK Pakistani patients. We used 100 anonymised samples from healthy UK Caucasians as controls for the patients with European origin. A mixture of 50 Caucasian and 50 Asian samples was used as controls for the Turkish and Tahitian patients with identified mutations. None of the presumed pathological DNA variants were found in the controls.

Estimating age of the most recent common ancestor

For estimating the age of the most recent common ancestor we applied a likelihood-based method, which uses multilocus marker data from a small number of patients suggested by Genin et al. (2004). We assume that all affected individuals in the sample descended from a common ancestor, who introduced the mutation n_{gen} generation ago. The problem is to estimate n_{gen} from the size of the haplotype shared by the individuals on each side of the disease locus D. The method is based on two functions: (a) the probability that no recombination occurred between D and marker M_x located at recombination fraction θ_x from D, $S(x) = (1-\theta_x)^n$ and (b) the probability that one crossing-over occurred in the interval between marker M_{x-1} and M_x, $f(x) = S(x-1)-S(x)$. Allele frequencies of the markers and mutation rates are taken into account to allow for the possibility that recombination occurred in previous intervals but with haplotypes sharing the same alleles as the ancestral haplotype or to let haplotype diversity be due to mutations rather than recombinations.

Marker allele frequencies were obtained from a sample of 30 unrelated controls from the same ethnic origin as the patients. Because genetic distances may not be accurate between closely linked markers, recombination fractions between disease locus and the different markers were obtained from the physical

distances between distant markers and their genetic distance in the Marshfield map. For the most distant markers of the map, D15S655 and D15S816, the physical distance was found to be approximately 7 Mb and the genetic distance (Marshfield) is 17.75 cM, giving 2.52 cM per Mb. However, when we considered separately each side of the mutation, very different results were obtained: from markers D15S655 to D15S127: we obtain 1.16 cM per Mb and from arc4 to D15S816, the correspondence is 4.20 cM per Mb. Therefore, analysis was performed using either the average 2.56 cM per Mb estimate or using the two side specific estimates and results were compared. The 95% confidence interval (95% CI) of the n_{gen} estimate was computed using a Bayesian approach.

Results

Clinical features of ARC syndrome

The clinical features from 62 children with ARC syndrome are presented in Table 1. As expected the four major diagnostic features of ARC syndrome (arthrogryposis, renal tubular dysfunction and cholestasis with a low gGT activity) were present in the vast majority of cases, but in addition, all patients failed to thrive (struggled to maintain birth weight). Almost half of the patients were born small for gestational age (30/62, 48%) and in 10 pregnancies oligohydramnios was reported. Parenteral nutrition was attempted in one patient (Denecke et al. 2000, pedigree 17) and some weight gain was achieved before the child died of overwhelming sepsis age 7 months. Elemental feeds were attempted in one child (pedigree 34), which did not prevent failure to thrive (increase in 10% of birth weight at 8 months), but the child is alive at the age of 8 months and has not suffered severe infections (pedigree 34). 17 patients (27%) were reported to have intermittent episodes of diarrhoea but this was not persistent or severe. All patients had conjugated jaundice and the level of bilirubin in an individual patient fluctuated between extremely high (300 μmol/l) and normal levels. Liver disease was associated with normal gGT and normal/slightly elevated ALT and AST (less than twice the upper limit of normal). Fourteen out of 15 studied patients had non-excreting biliary isotope studies suggesting biliary obstruction or severe intrahepatic cholestasis. Liver biopsy features included bile duct paucity in 7/16 and lipofuscin deposition in 5/16. In 12 patients giant cell transformation of hepatocytes associated with neonatal hepatitis was reported. These changes were relatively mild and not associated with lobular disarray. One patient developed acute liver failure during an episode of sepsis at the age of 6 weeks (pedigree 11), characterised by raised bilirubin and hepatic transaminases, and deranged coagulation.

All patients had a degree of renal tubular dysfunction with aminoaciduria, occasional glycosuria and renal tubular acidosis. The severity of renal tubular acidosis and hypernatraemic dehydration worsened during the episodes of intercurrent illness. Renal ultrasound (performed in 14 patients) and biopsy (performed in 3 patients) findings included poor cortico-medullary differentiation in 6 patients, nephrocalcinosis in 6 patients and tubular atrophy in 2 patients. Renal ultrasound scan was reported as normal in four cases.

Thrombocytopaenia and/or abnormal platelet morphology on light microscopy were identified in 7/62 patients. Seven out of 16 patients who underwent organ biopsies suffered life threatening or fatal haemorrhage and in further 4 patients there was spontaneous severe bleeding (pulmonary haemorrhage in 2, gastrointestinal in 2). Nine out of the above 11 patients with bleeding episodes had normal platelet count and morphology reported. Abnormal platelet function studies were found in 4 out of 4 studied patients (pedigrees 1, 6, 22, 30, see Table 1), one of whom had normal platelet count and morphology on light microscopy. None of the patients with abnormal platelet function had episodes of spontaneous bleeding, but one underwent organ biopsy and suffered severe haemorrhage.

Four patients had severe anaemia requiring blood transfusions.

Neurological abnormalities

Arthrogryposis was present in all but two patients (pedigree 2, p.Arg438X mutation), but the severity ranged from isolated talipes to severe forms including congenital hip dysplasia. Corpus callosum dysgenesis and other intracranial abnormalities were found in nine patients.

Other abnormalities

Ichthyosis was noted in most patients. Dysmorphic features included large hands, proximally inserted thumbs, low set ears, sloping forehead and hirsutism. Congenital cardiac anomalies included atrial septal defect (2), ventricular septal defect (2) and patent foramen ovale (2). Deafness was detected in 4 patients. 3 patients were found to have hypothyroidism.

Genotype-phenotype correlation in ARC

No clear genotype-phenotype correlations were identified (Table 2). Most children died in the first 6 months (median survival 5 months, range 1 week–20 months), but the two patients who survived more than 1 year had novel VPS33B mutations. Both were from non-consanguineous families. A homozygous frameshift mutation c.556_557delCT (p.Leu175fsX219) was detected in a non-consanguineous Italian patient (pedigree 18). Although this child had the classical triad of renal tubular dysfunction, cholestasis and arthrogryposis, he had no infections until the age of 14 months when he developed fatal pneumonia. In addition to a hypoplastic corpus callosum, he had other intracerebral abnormalities noted on the MRI of the brain such as an increased T1 weighted signal in the basal ganglia, capsular, corticospinal and Rolandic areas bilaterally. In view of the apparently mild phenotype in association with an exon eight frameshift mutation, we considered the possibility that his mutation might affect splicing and have a less-than-predicted effect on protein function. We used ''GENSCAN'' software program to look for the splicing signal in the genomic DNA (http://www.genes.mit.edu/GENSCAN.html) and this predicted that the two base pair deletion would create a new splice site earlier in the exon that would alter protein sequence encoded by exon 8 of the new protein from Start>>>>HLLSTL**YGPFPNCYGIGRCAK**MAYEL>>>Stop to Start >>>>>HLLSTL**WTLSKLLWNWQ**MAYEL>>>Stop, but thereafter the protein sequence would be unchanged.

A heterozygous splice donor site mutation (c.498+1 G>A) was identified in a child (pedigree 28) of non-consanguineous Swedish parents, who was born with severe renal tubular acidosis and cholestasis in the neonatal period. The patient had other typical features of ARC and also deafness and a hypoplastic corpus callosum. This child had severe failure to thrive despite nutritional supplementation and died at the age of 20 months due to pulmonary haemorrhage.

Evidence for a second ARC locus

Direct sequencing of all 23 exons and exon–intron boundaries of *VPS33B* gene did not identify any pathogenic changes in 7 (pedigrees 15, 17, 19, 23, 25, 26, 31) out of 35 families (20%). Patients from 3 of these families (15, 19 and 25), had typical features of ARC and came from non-consanguineous families that were unsuitable for linkage studies. In one family genotyping was consistent with linkage to VPS33B (pedigree 23), but three Turkish patients from consanguineous families (pedigrees 17, 26, 31) were heterozygous for VPS33B intragenic SNPs providing evidence for a possible second ARC locus.

Age of R438X mutation

The p.Arg438X mutation was identified in 12 Pakistani kindreds and we then genotyped 12 markers around the *VPS33B* gene (six markers on each side) in 8 patients who were not knowingly related (pedigrees in the families were traced at least 4 generations back, Table 3). The results were consistent with a common founder mutation and we then used the method of Genin et al. (2004) to estimate mutation age. Thus we performed two age estimations using either the average correspondence (2.52 cM per Mb) or using the two

Table 3 Haplotype analysis for the *ARC* region in patients with R438X mutation in *VPS33B*

Marker	Genetic distance	Allele frequency	Patient Physical distance	1	2	3	4	5	6	7	8
D15S979	93.21 cM	0.1	86562128	1	2						
D15S526	N/A	0.07	88117110	1	1	2					
D15S183	96.08 cM	0.2	88257073	1	1	1		2			
D15s996	N/A	0.16	88717135	3	3	3	3/4	2	1	2	2
arc2*	N/A	0.15	88997844	2	2	2	2	2	2	2	1
D15S127	97.26 cM	0.1	89127510	1	1	1	1	1	1	1	1
mutation	p.Arg438X		89275210								
arc4*	N/A	0.2	89356465	1	1	1	1	1	1	1	1
D15S158	98.27 cM	0.13	89471432	3	3	3	3	1/2	1/3	1/2	1/2
D15S963	N/A	0.03	89521931	3	3	3	3				
D15S652	99.92 cM	0.05	90247135	3	3	3	1				
D15s130	108.21 cM	0.15	92440938	1	1	1					
D15s816	110.93 cM		92749552	3	1	2/3					

Physical distance in basepairs. Genetic distance in centiMorgans as in the deCODE genetic map. *N/A* the position on the map is not available

different correspondences on each side. (1) With 2.52 cM per Mb the age estimate is 39 generations (95% CI 23–70), when assuming a negligible mutation rate at the markers. If the mutation rate is 10^{-4} per marker per generation, the age estimate is 38 (95% CI 22–69).

(2) With the two different distance correspondences on each side of the mutation (from markers D15S655 and D15S127: 1.16 cM per Mb and on the other side: 4.20 cM per Mb), the age estimate is 35 generations (95% CI of 20–62). As before, these results are not significantly affected by mutation rates. If we assume a mutation rate of 10^{-4}, the age estimate is 34 generations (95% CI 20–61). This shows that estimates are concordant with the two sets of recombination fractions and if we assume that one generation is 25 years, then the common ancestor should be about 900–1,000 years old (95% CI 500–1,525).

Discussion

VPS33B encodes a 617 amino acid protein that is a homologue of yeast Vps33p, a class C vacuolar protein sorting (vps) protein known to be involved in multiple stages of a vesicular trafficking pathway. VPS33B belongs to the Sec1/Munc18 (SM) family of proteins that are known to be involved in SNARE-dependent vesicle targeting and fusion (Gissen et al. 2005). Immunostaining of renal and liver biopsy material from ARC patients identified mislocalisation of several apical membrane proteins (Gissen et al. 2004; Bull et al. 2006). Interestingly, mislocalisation of the MDR1 protein was also found in hepatocytes of zebrafish *Vps18* mutant. Vps18 protein is also a class C vps protein, whose function is closely associated with Vps33 (Sadler et al. 2005). These findings suggest that class C vps proteins are involved in apical protein transport. Liver disease in ARC patients leads to cholestasis and bile duct paucity and knockdown of the *vps33b* ortholog in zebrafish embryo also leads to the bile duct paucity (Matthews et al. 2005). Thus VPS33B appears to be important in both function and development of the biliary tracts. Interestingly, embryonic expression of the zebrafish vps33b is particularly prominent in the liver and intestine but not in the kidneys, which is reflected in the fact that the vps33b knockdown zebrafish had no renal functional or morphological defects (Matthews et al. 2005).

We have characterised the clinical and molecular features of a cohort of 62 patients with a clinical diagnosis of ARC syndrome from 14 different ethnic backgrounds. All patients had severe failure to thrive (difficulty to maintain birth weight) and most died in the first 6 months of life. The severity of the failure to thrive observed exceeded that expected for the degree of liver and intestinal dysfunction and poses the question about the cause for such growth restriction. The identification of mislocalised apical membrane proteins suggests a possibility of a global abnormality in intestinal absorption and renal tubular reabsorption. Further research may identify the functional defect and collaborative studies could provide objective evidence for the use of special feeds which may overcome the reduced absorption of nutrients.

Most patients with ARC are reported to have skin ichthyosis. Interestingly some of the clinical features found in ARC patients overlap with those in the recently described syndrome cerebral dysgenesis, neuropathy, ichthyosis and keratoderma (CEDNIK) associated with a mutation in SNAP29 gene, coding for a SNARE protein (Sprecher et al. 2005). Patients with CEDNIK have severe ichthyosis associated with abnormal maturation and fusion of the lamellar bodies. Secretion of lamellar bodies is critical for epidermal cohesion and waterproofing (Elias et al. 2003). Lamellar bodies represent a type of secretory vesicles, which belong to a class of lysosome-related organelles analogous to the lamellar bodies of alveolar type 2 cells and also platelet alpha granules, found to be deficient in the ARC patients (Lo et al. 2005). Abnormal synthesis and function of lamellar bodies could be the cause of ichthyosis in ARC as VPS33B is involved in regulation of vesicular membrane fusion by interacting with SNARE proteins. Alternatively, abnormal intestinal absorption leading to nutritional deficiencies could also result in ichthyosis. Other similarities between ARC and CEDNIK include brain MRI findings and neurogenic atrophy found on muscle biopsy. Linkage to the *CEDNIK* locus was excluded in some ARC patients unlinked to the *VPS33B*.

Only a small number of patients survived beyond 1 year and it is possible that this might reflect partial retention of VPS33B function. The severity of arthrogryposis was variable even amongst those with the common p.Arg438X mutation. A small number of patients with ARC without clinically detected arthrogryposis have been reported elsewhere (Coleman et al. 1997; Bull et al. 2006). However, mutation analysis for patients reported by Coleman et al. (1997) was not available. Bull et al. (2006) reports a patient with classical ARC features, who had rocker-bottom feet but no arthrogryposis, homozygous for a mutation in *VPS33B* gene. We would have classified rocker-bottom feet as part of the arthrogryposis

spectrum. Patients in pedigree 2 (see Table 1) had no clinically detected arthrogryposis unlike their two siblings and three cousins. Although arthrogryposis in ARC syndrome may be partially neurogenic in origin, the degree of arthrogryposis may depend on the fetal position and severity of oligohydramnios (reported in ten pregnancies). As polyuria is one of the cardinal signs of ARC, the oligohydramnios could not be accounted for by a fetal renal abnormality, thus the cause of this phenomenon may be a placental insufficiency resulting in oligohydramnios and also intrauterine growth retardation (reported in 48% of cases) secondary to a protein trafficking defect (Sanseverino et al. 2006).

We note that approximately 10% of ARC patients had a structural cardiac defect implicating VPS33B-related vesicular transport in normal cardiac development. In view of the susceptibility of the ARC patients to infection, the diagnosis of congenital cardiac anomaly should be sought in all ARC patients. Finding such abnormality would make physicians aware of the possibility of bacterial endocarditis.

Grey platelet syndrome results from an abnormal biosynthesis and function of platelet alpha granules and there was a spectrum of severity in this disorder. There were frequent episodes of severe bleeding leading to premature death in this cohort. These episodes occurred in patients without any morphological platelet abnormalities and so normal routine platelet analysis will not identify the risk of severe bleeding in suspected ARC syndrome patients. We recommend that organ biopsies should not be performed as a diagnostic procedure for ARC syndrome but should be replaced by *VPS33B* mutation analysis. If this is negative, then organ biopsy should only be considered after detailed platelet function studies and support (Lo et al. 2005).

The identification of specific mutations which occur at higher frequency in different ethnic groups (e.g., p.Arg438X in Pakistanis, p.Arg507X in Portuguese) will facilitate mutation detection. Interestingly we dated the p.Arg438X mutation to 900–1,000 years ago and this may correspond to when the original Arabic and Muslim settlers came to Kashmir (Wolpert 2000). Identification of the further locus for ARC syndrome will facilitate molecular genetic diagnosis and may offer insights into VPS33B function.

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