Phenotypic variability in 49 cases of *ESCO2* mutations, including novel missense and codon deletion in the acetyltransferase domain, correlates with *ESCO2* expression and establishes the clinical criteria for Roberts syndrome

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ABSTRACT

Background Roberts syndrome (RBS) and SC phocomelia are caused by mutations in *ESCO2*, which codes for an acetyltransferase involved in the regulation of sister chromatid cohesion. Of 26 mutations described to date, only one missense mutation has been reported and all others are predicted to be truncating mutations. Genotype—phenotype analysis has been hampered by limited numbers of patients with clinical information available.

Objective To provide unpublished clinical data for 31 patients with proven *ESCO2* mutations and combine this series with previously reported clinical and mutation data on 18 cases.

Methods Genotype—phenotype correlations and functional effects of two novel *ESCO2* mutations were analysed. In situ hybridisation on human embryos at Carnegie stages 14, 17 and 21 was performed to study *ESCO2* expression during development.

Results and conclusions Using the cohort of 49 patients, the clinical criteria for RBS were delineated to include: growth retardation; symmetric mesomelic shortening of the limbs in which the upper limbs are more commonly and severely affected than the lower limbs; characteristic facies with microcephaly. The severity of malformations of the facies correlates with the severity of limb reduction. The occurrence of corneal opacities may be associated with specific mutations. Two new mutations, both in the *ESCO2* acetyltransferase domain, are described and their acetylation effects in vitro demonstrated. In situ hybridisation on human embryos showed *ESCO2* expression in the brain, face, limb, kidney and gonads, which corresponds to the structures affected in RBS.

Roberts syndrome (RBS; MIM 268300) is an autosomal recessive developmental disorder, characterised by growth retardation, limb reduction and craniofacial abnormalities including cleft lip and palate, which was described in 1919 by John Roberts in affected siblings from a consanguineous Italian couple.[1] Fifty years later in 1969, SC phocomelia (MIM 269000) was reported as a similar but milder syndrome by Herrmann *et al*[2] in two families of European descent. Then, 30 years ago in 1979, Tomkins *et al*[3] described a characteristic cytogenetic defect or centromere puffing, affecting most of the chromosomes in all metaphases in RBS and SC phocomelia. James German[4] used the term premature centromere separation (PCS) or RS (Roberts syndrome) effect.

The causative gene for RBS was found to be *establishment of cohesion 1 homologue 2 (ESCO2).*[5] Later, on the basis of analysis of eight unrelated patients, it was proposed that RBS and SC phocomelia are allelic disorders caused by mutations in *ESCO2*.[6] To date, 26 different mutations in *ESCO2* have been reported.[5–8] Most are predicted to result in protein truncation or mRNA instability, and only one missense mutation and one in-frame 48-amino acid deletion have been found. Owing to the scarcity of missense mutations and small in-frame insertions or deletions, it was proposed that different clinical phenotypes may result from *ESCO2* missense mutations.[6]

ESCO2 encodes a 601-amino acid protein belonging to the Eco1 family of acetyltransferases involved in the establishment of sister chromatid cohesion during S phase and postreplicative sister chromatid cohesion induced by double-strand breaks.[9–11] It has been proposed that establishment of cohesion might be regulated directly or indirectly by the acetylation activity of these proteins.[9 12] Concordantly, loss of ESCO2 acetyltransferase activity was recently implicated in the molecular mechanism of RBS.[8]

Here we report on two novel changes in *ESCO2*: one missense, a putative causative mutation, and a single-codon deletion. We characterise the associated severity and spectrum of phenotypic features of 49 patients, 31 of whom had no previously published clinical information. In addition to providing data on well-established features, we describe a number of less common findings. Our study, the largest reported to date, allows us to establish a set of clinical criteria for patients with *ESCO2* mutations. We also report that the expression of *ESCO2* during human embryo development correlates with the structures affected in RBS/SC phocomelia.

METHODS

Patients

A clinical questionnaire was completed for 31 patients by clinical geneticists and from medical

chart review after consent had been obtained and in accordance with institutional review board guidelines. These patients belonged to 26 families of different origins (supplementary table 1 online). In addition, a literature review was performed on 18 patients to include clinical data of all patients with *ESCO2* mutations to date (supplementary table 2 online).[2] [4–8] [13–20] Additional phenotypic information from previously reported patients was incorporated when available from medical charts. Cytogenetic analysis revealing PCS was available for all patients except patients 3275 and 3349.

Molecular analysis of *ESCO2*

ESCO2 mutations were previously published for most cases.[5–8] [20] For patients 62101 and fetus A and B from family 26, *ESCO2* mutations were analysed as previously reported.[8]

ESCO2 autoacetyltransferase activity

Recombinant C-terminal domains of ESCO2 wild-type, E453del and G581R were produced as MBP-His fusion proteins in *Escherichia coli*. After purification, incubation with acetyl-CoA and sodium dodecyl sulfate polyacrylamide gel electrophoresis, protein acetylation was detected by western blot analysis using an antibody against acetyl-lysine (Cell Signaling Technology, Beverly, Massachusetts, USA). The amount of protein loaded was detected using a His antibody.

ESCO2 expression in human embryos

The human embryo sections were provided by the Joint MRC-Wellcome Human Developmental Biology Resource at IHG, Newcastle upon Tyne, UK. Sense and antisense probes from *ESCO2* were synthesised by transcribing and labelling linearised plasmid (pGEMTeasy) containing fragments (nucleotides 1163–1889 and nucleotides 1343–1889) of GenBank accession number AY882862 with T7 and SP6 RNA polymerase using the Dig RNA labelling kit (Roche Diagnostics Ltd, Burgess Hill, UK). The probes were hybridised to the tissue-section slides, washed and developed as described by Moorman *et al*.[21]

Statistical analysis

Genotype–phenotype correlations and phenotypic associations were performed using contingency tables. Statistical difference was tested with the Fisher exact test (2×2 or 2×3). The McNemar test was used for paired proportions. $p < 0.05$ was considered significant. All statistical tests were computed using SPSS V12.0 or the calculators at VassarStats (http://faculty.vassar.edu/lowry/VassarStats.html).

RESULTS

Identification of new mutations in *ESCO2*

Sequence analysis of the coding sequence of *ESCO2* revealed a novel homozygous mutation in family 23. The mutation corresponds to the small deletion c.1359_1361delAGA (reference sequence NM_ 001017420) in exon 9 (supplementary figure 1 online). This mutation results in the in-frame deletion of a glutamic acid at position 453 (p.E453del). Analysis of parental DNA showed the deletion in a heterozygous state in both parents. Cells were not available for further RNA and protein studies.

In family 26, there was a history of two pregnancies with affected fetuses, but no samples were available. Only parental DNA was studied. Sequence analysis of maternal DNA revealed a heterozygous G to C transversion at position 1741 in exon 11 as the putative disease-causing mutation (supplementary
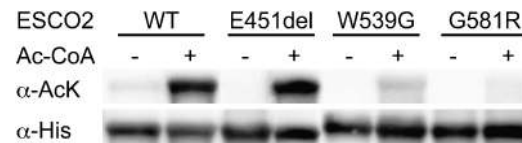

Figure 1 Autoacetylation activity of wild-type (WT) and mutant ESCO2. For each protein, a C-terminal domain fragment containing the C2H2 zinc finger and acetyltransferase domains was tested. In the upper panel, autoacetylation was analysed by western blotting using an antibody against acetylated lysine (α-AcK). An equal amount of each protein was confirmed by probing the membrane with an antibody against the His tag fused to ESCO2 (α-His, lower panel). The previously reported W539G mutant was included as a control.[8] Ac-CoA, acetyl-coenzyme A.

figure 1 online). This missense mutation results in the substitution of a highly conserved glycine by an arginine at position 581 (p.G581R) in the acetyltransferase domain. The father's DNA had no detectable *ESCO2* mutations. Unfortunately, additional samples were not available from the father for further analysis of non-coding regions, dosage and transcription. Owing to the lack of fetal samples, paternity was not confirmed and uniparental disomy could not be tested. Cytogenetic analysis of the pregnancies confirmed premature separation of the centromeres.

In vitro acetyltransferase activity of mutated ESCO2

To investigate the effect of the E453del and G581R mutations on ESCO2 function, we tested the in vitro autoacetyltransferase activity of recombinant mutated protein produced in *E coli*. The ESCO2 G581R mutation resulted in decreased acetyltransferase activity of the recombinant protein, whereas no reduction was observed with ESCO2 E453del (figure 1).

Clinical description

Clinical findings in patients with the two new *ESCO2* mutations as well as clinical data for 47 patients with known *ESCO2* mutations are presented (supplementary tables 1 and 2 online). Table 1 gives a summary of 33 major findings in all cases. In total, there were 39 families with 31 males and 18 females affected. All patients, except six fetuses with limited clinical information, presented with growth retardation, limb reduction and craniofacial abnormalities. More than 50% of the cases had mental retardation, microcephaly, exophthalmos, hypertelorism, ear malformation, cleft lip and palate, brachydactyly and clinodactyly. Brachycephaly, midfacial haemangioma, down-slanting palpebral fissures, hypoplastic nasal alae, malar hypoplasia and micrognathia also commonly occurred (figure 2), and this has not been stressed previously. More than 80% of patients had both upper and lower extremities affected.

When this cohort with *ESCO2* mutations was compared with the cohort of RBS patients with PCS/heterochromatin repulsion (HR) reported before the knowledge of the disease gene, the frequencies of common features were similar (table 1).[16] We only observed a significant difference for cardiac defects ($p = 0.005$). Thus, this large cohort further validates that PCS/HR and *ESCO2* mutations are highly correlated in RBS.

Besides the common abnormalities described in table 1, a number of additional abnormalities were found (table 2).

Genotype–phenotype correlation and phenotypic associations

Genotype–phenotype analysis revealed no clear correlation between clinical findings and the type of mutation, inferred length of the mutant protein, or mutations in specific domains of

Table 1 Clinical features in Roberts syndrome/SC phocomelia patients with *ESCO2* mutations in this study as compared with those with premature centromere separation (PCS)/heterochromatin repulsion (HR) reported before *ESCO2* identification

	ESCO2 mutations*		PCS/HR + Van den Berg and Francke[16] modified†	
	49 Cases‡	Frequency (%)	33 Cases‡	Frequency (%)
Gender	31M, 18F	1.72 ratio	17M, 14F, 2U	1.25 ratio
Growth retardation	45/45	100%	26/28	92.9%
Mental retardation/ developmental delay	20/34	58.8	12/17	70.6
Craniofacial abnormalities				
Brachycephaly	19/30	63.3	NR	NR
Microcephaly	38/40	95.0	19/23	82.6
Midfacial haemangioma	25/37	67.6	10 cases	NR
Exophthalmos	25/42	59.5	14/17	82.4
Hypertelorism	36/42	85.7	16/21	76.2
Down-slanting palpebral fissures	17/31	54.8	NR	NR
Corneal opacities	13/36	36.1	8/11	72.7
Ear malformation	23/35	65.7	12/16	75
Hypoplastic nasal alae	36/39	92.3	10 cases	NR
Malar hypoplasia	29/33	87.9	NR	NR
Cleft lip and palate	24/44	54.5	10/29	34.5
Cleft palate	1/44	2.3	2/29	6.9
Micrognathia	29/39	74.4	6 cases	NR
Limb abnormalities				
Phocomelia	49/49	100	31/33	93.9
Arms and legs	39/48	81.2	29/33	87.9
Only arms	9/48	18.8	2/38	5.3
Humeral defects	32/41	78.1	14/18	77.7
Hypoplasia	28/41	68.3		
Aplasia	4/41	9.8		
Ulnar defects	41/42	97.6	24/27	88.9
Hypoplasia	24/42	57.1		
Aplasia	17/42	40.5		
Radial defects	43/43	100.0	28/30	93.3
Hypoplasia	15/43	34.9		
Aplasia	28/43	65.1		
Thumb defects	44/45	97.8	NR	NR
Hypoplasia	14/45	31.1		
Aplasia	30/45	66.7		
Number of fingers¶	31.46	67.4	22/26	84.6
Brachydactyly	23/33	69.7	NR	NR
Clinodactyly	23/31	74.2	NR	NR
Arm bone synostosis	12/25	48	2 cases	NR
Femoral defects	23/40	57.5	13/21	61.9
Hypoplasia	21/40	52.5		
Aplasia	2/40	5.0		
Tibial defects	29/42	69	21/29	72.4
Hypoplasia	25/42	59.5		
Aplasia	4/42	9.5		
Fibular defects	31/42	73.8	24/31	77.4
Hypoplasia	15/42	35.7		
Aplasia	16/42	38.1		
Number of toes¶	3/40	7.5	6/22	27.3
Club foot	21/33	63.6	NR	NR
Other abnormalities				
Enlarged phallus	7/23	30.4	7/15	46.7
Enlarged clitoris	5/11	45.5	NR	NR
Cryptorchidism	4/18	22.2	4/11	36.4
Cardiac defect	8/31	25.8	9/12	75.0

*All known cases with *ESCO2* mutations also have PCS/HR.

†We excluded from the analysis in the last two columns the five cases for which *ESCO2* mutations were reported after Van den Berg and Francke's review[16] and that are included in the first column, "*ESCO2* mutations".
‡Numerator represents the number of cases with positive findings, denominator represents number of cases with information available.
¶Number of fingers or toes refers to cases with fewer than five fingers or toes.
NR, not reported.

ESCO2. However, we observed that the presence or absence of corneal opacities segregated with specific mutations. All seven individuals in four families with the 750_751insG mutation and six in six families with the 879_880delAG mutation did not have corneal opacities, whereas all five affected with the mutation 505C>T or 1597_1598insT had corneal opacities. We also observed that 11 patients without corneal opacities having the 750_751insG or 879_880delAG mutation did not present with cardiac abnormalities, suggesting a possible association between these two phenotypic features. Indeed, when we analysed the total cohort, we found that patients without corneal opacities were less likely to present with cardiac abnormalities ($p = 0.0022$) (table 3). In addition, we found that patients with corneal opacities were more likely to present with mental retardation ($p = 0.0006$). We also observed that patients with mental retardation were more likely to present with cardiac defects. However, this association is not significant at the $\alpha = 0.05$ ($p = 0.073$).

We also evaluated the association between skeletal abnormalities and other clinical features (table 3). We found that reduction of both arms and legs was more common in patients with cleft lip and palate ($p = 0.015$). In addition, humerus, fibula, tibia and femur abnormalities were more common in patients with cleft lip and palate ($p = 0.013$, $p = 0.025$, $p = 0.004$ and $p = 0.008$, respectively). The association between radius and ulna defects with the presence of clefts was not statistically significant, but aplasia rather than hypoplasia of these bones appeared to be more common in the presence of clefts. Patients with cleft lip and palate were also more likely to have only three fingers,
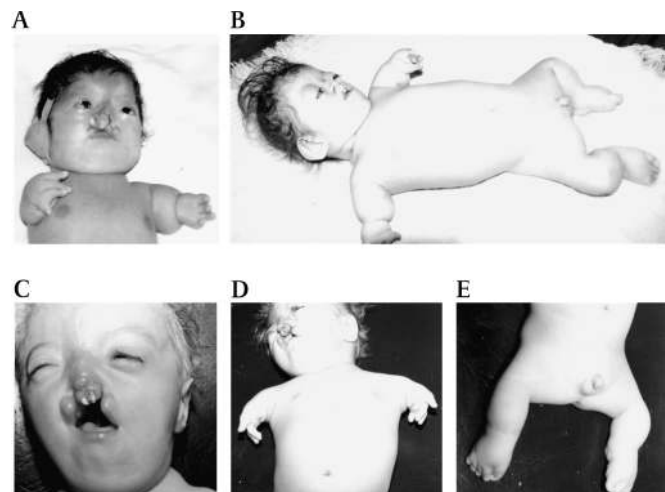

Figure 2 Photographs of patients with Roberts syndrome (RBS). (A,B) Patient with the *ESCO2* c.505C>T mutation in the neonatal period (A) and 1 year later (B). (C,D,E) Patient with *ESCO2* c.879_880delAG mutation. (A,C) RBS facies with microcephaly, hypoplastic nasal alae, malar hypoplasia, hypertelorism, micrognathia, haemangioma, exophthalmos, down-slanting palpebral fissures and cleft lip and palate. (B,D,E) RBS limb reduction is mesomelic, symmetric and follows a cephalocaudal pattern in which the arms are more severely affected than the legs.

Table 2 Additional clinical findings in 49 patients with ESCO2 mutations

	No of cases (%)
Ophthalmological findings	
Bluish sclerae	11 (22)
Microphthalmia	4 (8)
Glaucoma	4 (8)
Nystagmus	4 (8)
Optic atrophy, miotic poorly reactive pupil, Peter's anomaly, presbyopia, slightly blurred disk margins without papilloedema, tilting of the right optic disc	1 (2)
Additional craniofacial findings	
Highly arched palate (in patients that did not have cleft palate)	5 (10)
Sparse silvery blond hair	4 (8)
Craniosynostosis	2 (4)
Short neck	2 (4)
Torticollis, frontal encephalocele, plagiocephaly, nasolacrimal duct stenosis, blepharophimosis, partial agenesis of the corpus callosum, bilateral peripheral 7th cranial nerve weakness, paralysis of the left side of the soft palate, bilateral facial weakness, hearing problems, absence of the vomer and the nasal septum, deviated nasal septum	1 (2)
Additional limb findings	
Syndactyly	9 (18)
Flexion contractures	8 (16)
Metacarpal fusion	6 (12)
Femorotibial synostosis	4 (8)
Wide gap between first and second toe	4 (8)
Transverse palmar creases	3 (6)
Absent or hypoplastic patella	2 (4)
Skeletal findings	
Bilateral hypoplastic scapulae, anteriorly rotated scapulae, small vertebra with thoracic kyphoscoliosis, absence of the pubic rami of the pelvis, partial sacral agenesis, open sacral dimple, presence of only 11 ribs	1 (2)
Café-au-lait spots in trunk and extremities	5 (10)
Seizures	3 (6)
Stroke	2 (4)
Arterial occlusion	1 (2)
Myocardial infarction (present at 23 years)	1 (2)
Cavernous haemangioma of the optic nerve	1 (2)
Moya-Moya disease	1 (2)
Malign melanoma	1 (2)
Cystic dysplasia of the right kidney	1 (2)
Unilateral hydronephrosis	1 (2)

In rows with multiple features, the corresponding number represents the number of patients with each of the features.

whereas patients without clefts were less likely to have reductions in the number of fingers (p = 0.003).

ESCO2 expression in human embryos

We performed in situ hybridisation on human embryos at Carnegie stages (CS) 14, 17 and 21 (approximately 32, 41 and 52 days after ovulation, respectively). We found that ESCO2 was expressed in the brain, first and third branchial arches, otocyst, dorsal root ganglia, limb buds, kidney and gonads (figure 3). At CS 14, ESCO2 expression was detected in the neuroepithelium of the hindbrain, midbrain, telencephalic vesicle (forebrain), otocyst, mandibular component of the first and third branchial arches and developing dorsal root ganglia. At the limb buds, the ESCO2 expression showed a homogeneous mesenchymal expression pattern at CS 14. Interestingly, a separate section also indicated more peripheral, possibly ectodermal expression. At CS 17, the expression in limbs became confined to discrete zones in the developing hand plate. At CS 21, the expression of ESCO2 appeared to be confined to areas surrounding the distal tip of

**Table 3** Phenotypic associations in patients with ESCO2 mutations

Phenotype			p Value*
Corneal opacities, cardiac defect and mental retardation			
	Corneal opacities		
	+	−	
Mental retardation	100.0% (12/12)	36.4% (8/22)	0.0006
Cardiac defect	75.0% (6/8)	10.0% (2/19)	0.0022
	Mental retardation		
	+	−	
Cardiac defect	46.2% (6/13)	8.3% (1/12)	0.073
Limb and craniofacial abnormalities			
	Cleft lip and palate		
	+	−	
Midfacial haemangioma	47.3% (9/19)	87.5% (14/16)	0.030
Humeral hypoplasia/aplasia	95.2% (20/21)	58.8% (10/17)	0.013
Fibular hyoplasia/aplasia	90.5% (19/21)	48.5% (10/18)	0.025
Tibial hypoplasia/aplasia	90.5% (19/21)	44.4% (8/18)	0.004
Femoral hypoplasia/aplasia	80.0% (16/20)	33.3% (6/18)	0.008
Phocomelia	95.7% (22/23)	63.2% (12/19)	0.015
Thumb			
Hypoplasia	13.6% (3/22)	47.4% (9/19)	0.016
Aplasia	86.4% (19/22)	47.4% (9/19)	
Number of fingers			
5	15.0% (3/20)	52.6% (10/19)	0.003
4	50.0% (10/20)	47.4% (9/19)	
3	35.0% (7/20)	0.0% (0/19)	
	Exophthalmos		
	+	−	
Arm bone synostosis	76.9% (10/13)	10.0% (1/10)	0.001
Number of fingers			
5	20.8% (5/24)	41.2% (7/17)	0.025
4	45.8% (11/24)	58.8% (10/17)	
3	33.3% (8/24)	0.0% (0/17)	
	Hypertelorism		
	+	−	
Fibular hyoplasia/aplasia	75.8% (25/33)	20.0% (1/5)	0.012
Tibial hypoplasia/aplasia	81.8% (27/33)	20.0% (1/5)	0.027
Thumb			
Hypoplasia	32.4% (11/34)	0.0% (0/6)	0.02
Aplasia	67.6% (23/34)	83.3% (5/6)	
	Down-slanting palpebral fissures		
	+	−	
Brachydactyly	86.7% (13/15)	33.3% (4/12)	0.004
	Malar hypoplasia		
	+	−	
Brachydactyly	76.9% (20/26)	0.0% (0/24)	0.008
Clinodactyly	80.8% (21/26)	25.0% (1/4)	0.019
	Micrognathia		
	+	−	
Tibial hypoplasia/aplasia	81.5% (22/27)	40.0% (4/10)	0.014

There are two sub-tables "Corneal opacities, cardiac defect and mental retardation" and "Limb and craniofacial abnormalities". In the first sub-table, for example, 12 patients had corneal opacities (+) and all 12 had mental retardation, while 22 patients did not have corneal opacities (−) and eight of them had mental retardation. The association of corneal opacities and mental retardation was significant (p = 0.0006). The denominators vary depending on whether clinical information is available for the presence or absence of specific features.
*Except for the association between mental retardation and cardiac defect, only significant associations at p = 0.05 are shown.

developing cartilaginous bone of the long bones of the forearm, wrist and phalanges and underlying the developing sternum. In kidney, expression of ESCO2 at CS 21 was localised to the metanephric cortex. At this stage, expression was also detected in neighbouring male gonadal epithelium. There was absence of

Figure 3 *ESCO2* expression in human embryos. (A) Sagittal section at Carnegie stage (CS) 14. Upper left panel shows expression on the developing brain, lower limb and branchial arches. In the right panels, magnified views show expression in the neuroepithelium of the hind brain (a), midbrain (b), telencephalic vesicle (forebrain) (c), otocyst (d), mandibular component of the first (e) and third (f) branchial arches and dorsal root ganglia (g). Lower left panel shows a section hybridised with the sense control. (B) Sagittal section at CS 17. Expression was present in the neuroepithelium in the roof of the midbrain (a), the hindbrain (b), facial mesenchyme (c) and developing lower jaw (d). Lower right panel shows the sense control section. (C) Transverse section at CS 21 shows expression in the gonadal ridge (a), metanephric cortex (b), dorsal root ganglia (c) and spinal cord (d). (D) *ESCO2* expression shows a homogeneous mesenchymal expression pattern in the sagittal section of limb at CS 14. In the lower right panel, a separate section (magnified from top left section in (A) and labelled with an asterisk) indicates more peripheral expression. (E) Sagittal section at CS 17. Expression was detected in discrete zones within the hand plate. (F) Transverse section at CS 21. Expression appears to be confined to areas surrounding cartilaginous bone in distal rather than proximal regions.
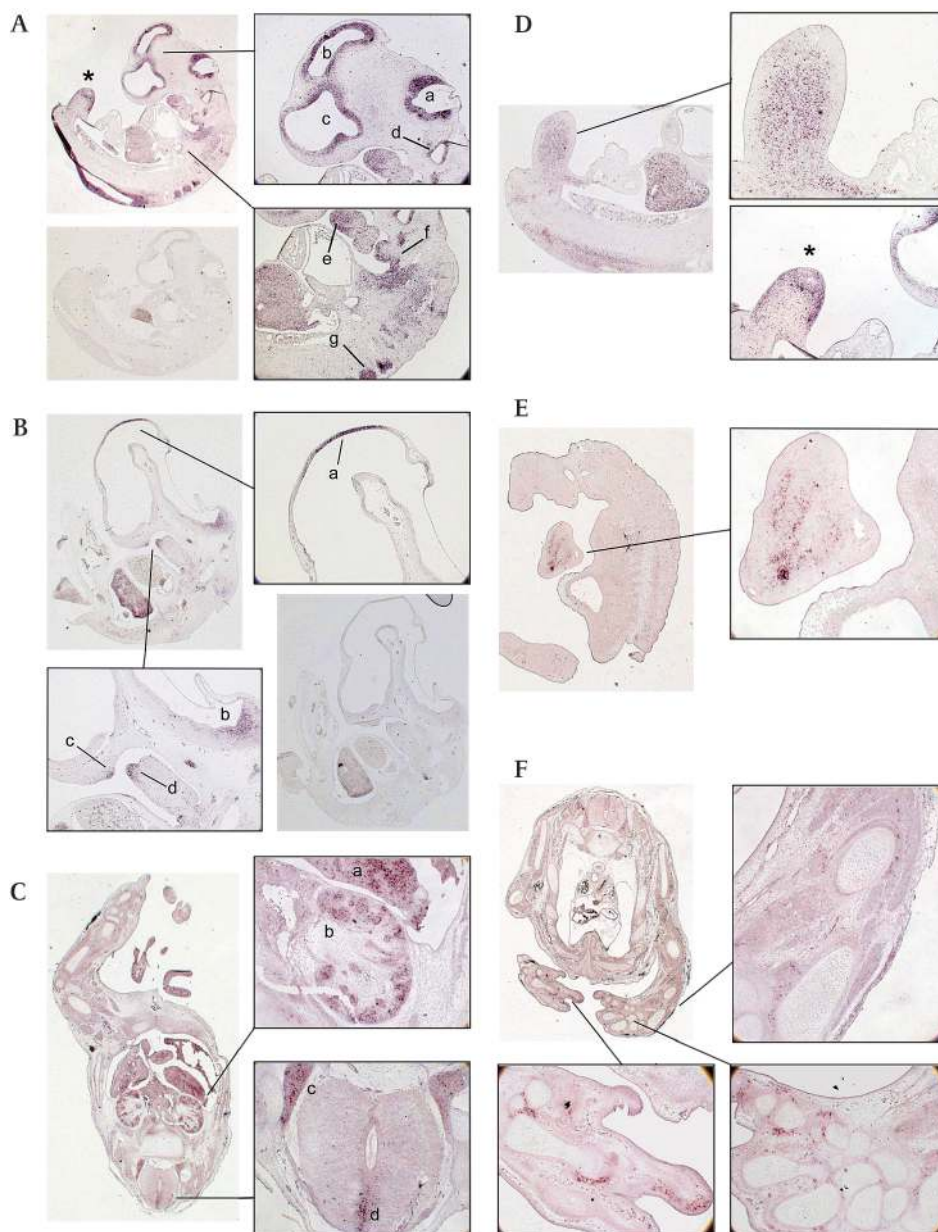
detectable expression in the eye, surrounding vertebral body and ribs, and cardiac tissues and developing great vessels at the stages tested (data not shown).

DISCUSSION

The majority of mutations in RBS described to date involve introduction of a new premature stop codon in the *ESCO2* transcript by frameshift, nonsense or splicing mutations, resulting most probably in either decreased mRNA levels due to nonsense-mediated decay or, if protein is produced, in a shorter protein product.[5–8 20] We have identified two novel single-amino acid alterations in ESCO2 in families affected with RBS/SC phocomelia: a deletion that targets the codon AGA, which encodes a glutamic acid at position 453 in exon 9, and a transversion in exon 11 that results in the substitution of an arginine for a glycine at position 581. These mutations, as well as the W539G missense mutation described previously, are all located in the C-terminal portion of ESCO2, which is the region that has similarity to acetyltransferases of the GNAT superfamily.[5 9]

We found that the E453del mutation does not affect the autoacetyltransferase activity of the C-terminal portion of ESCO2. Although E453 is located at the carboxy end, which contains the putative acetyltransferase domain, this amino acid is located outside the core region proposed to be shared by the Eco1 family and the GNAT superfamily. The core region of similarity includes motifs D, A and B of the GNAT fold, with the Eco1 family apparently lacking motif C and the α2 helix of the histone acetyltransferase structure.[9] However, the absence of sequence similarity does not necessarily exclude the existence of a functional motif C or α2 helix in the Eco1 family. On the basis of sequence similarity, motif C was initially thought to be missing in the histone acetyltransferases of the GNAT family,[22] but subsequent crystal structure analysis showed structural homology in this region and the presence of motif C in hPCAF, tGCN5 and yGCN5.[23–25] Residues in motif C and the α2 helix of tGCN5 have been implicated in substrate-binding specificity.[23] Secondary structure analysis of ESCO2 with JPRED predicts an α helix, with E453 in the middle of the helix, in the region N-terminal to motif D which may correspond to the α1

Box 1 Major characteristics of Roberts syndrome (RBS)

▶ Limb reduction is symmetric and follows a cephalocaudal pattern in which the arms are more commonly and severely affected than the legs (table 1, figure 2 and supplementary table 1). We found nine cases with only upper limb anomalies, but none where only the lower limbs were affected. Significant differences were observed between ulna and tibia and humerus and femur abnormalities (p<0.003, p = 0.008, respectively), and the radius was affected in all the patients, whereas only 73.8% of patients presented with fibular defects. Similarly, there were missing fingers in 67.4% of cases, mostly due to thumb aplasia, while missing toes were observed in just 7.5% of the cases. The degree to which the arms were affected correlated with lower limb abnormalities in that there was no patient with severe arm reduction abnormalities with normal legs, and, in the cases where only arms were affected, the reduction was moderated.

▶ In RBS, limb reduction affects the distal—proximal and anterior—posterior axes, resulting in a mesomelic reduction with a hand-specific affection pattern in which the thumb is always the first finger being affected (figure 2). We found that, in the upper limbs, the radius was always affected, followed in frequency by the ulna (97.6%) and the humerus (78.1%) (table 1). Hands were characteristically affected, with 97.8% of the cases affected with either aplasia (66.7%) or hypoplasia (31.1%) of the thumbs. Other fingers were affected at a lower frequency. In the lower limbs, the fibula was the bone most commonly and severely affected (73.8%), followed by the tibia (69%) and the femur (57.5%).

▶ There is a distinct RBS facies conferred by characteristic craniofacial abnormalities that include microcephaly, hypoplastic nasal alae, malar hypoplasia, hypertelorism, micrognathia, haemangioma, exophthalmos, down-slanting palpebral fissures and cleft lip and palate (figure 2). We want to emphasise that microcephaly is one of the most common characteristics of this syndrome (95%). We found that microcephaly in males was more severe than in females and also severity was more marked after the perinatal period (supplementary figure 2 online). In the present cohort, the two patients without microcephaly correspond to a male premature stillborn of unknown gestational age with no measurement available and a male fetus of 30 weeks of gestation.

▶ There is a correlation between the degree of limb and facial malformations. Although some of the major craniofacial characteristics have previously been proposed to be associated with severity of the phenotype, we found that only the presence of cleft lip and palate was associated with severe abnormalities in arms and legs (table 3).[6] [27] The presence of other facial abnormalities was associated with only some limb abnormalities to a statistically significant level. We suggest that the degree of severity of some facial abnormalities, which is difficult to quantitate, may correlate with the degree of affection of the limbs.

helix in motif C or the α2 helix in other members of the superfamily (data not shown).[26] Without knowledge of the structure of ESCO2 and its orthologues, it is not possible to know whether the E453del mutation perturbs the structure of a putative motif C, causing defective substrate interaction or recognition that may not be detected by the in vitro autoacetylation assay.

Alternatively, the E453 deletion may result in unstable ESCO2 or disrupt protein—protein interactions necessary for the regulation of ESCO2. The latter possibility, leading to misregulation or ectopic ESCO2 acetyltransferase activity in vivo, may result in abnormal clinical and cytogenetic phenotypes in the presence of normal in vitro enzyme activity. Indeed, Eco1, the budding yeast ESCO2 homologue, is proposed to be the hub for the regulation of cohesion establishment.[11]

Loss of ESCO2 enzymatic activity caused by the W539G mutation has been implicated in the pathogenesis of RBS.[8] We found that the novel missense mutation G581R reduced significantly the autoacetyltransferase activity in vitro. Gly581 is a highly evolutionarily conserved residue that is located at the beginning of the putative α4 helix in motif B of the ESCO2 acetyltransferase domain. Compared with glycine, arginine is a much bulkier molecule with a polar positively charged side chain, and therefore this substitution would be expected to interfere with normal helix formation and stability resulting in impaired enzymatic activity. Although we did not find any pathogenic or non-pathogenic mutation in the father's DNA after direct sequencing of the entire ESCO2 cDNA, the fact that the G581R mutation has a similar effect on in vitro autoacetyltransferase activity to the W539G mutation supports the notion that G581R contributes to RBS in this family.

The literature differentiates between RBS and SC phocomelia. This report, the largest phenotype analysis of affected individuals with proven ESCO2 mutations reported, allowed us to observe for the first time that these two conditions can be caused by the same mutation in different members of the same family rather than just in unrelated families. In particular, marked intrafamilial and interfamilial variability was observed in families affected by the mutations 505C>T, 750_751insG and 879_880delAG (table 1). In each of these families, there are members with phenotypes resembling the patient originally described by Roberts[1] (eg, R31, R5 and KAT) and members with phenotype similar to that described by Herrmann[2] as SC phocomelia (eg, R12, R27 and VU0288). We propose that hereafter the phenotypic spectrum associated with ESCO2 mutations be called Roberts syndrome, the first name associated with this condition. In addition, our data indicate that the clinical phenotype associated with ESCO2 missense mutations is equivalent to that produced by other types of mutation. Recent evidence supports this notion, as the W539G missense mutation was found to produce the same cellular phenotypes as that seen with nonsense and frameshift mutations.[8]

Our cohort covers a wide range of severity and therefore allows a clear delineation of the phenotype. In box 1, we suggest the major characteristics of RBS, some of which have previously been proposed.[6] [27] These clinical criteria may assist in differentiating between RBS and other tetraphocomelia and amelia conditions that share some clinical features but have different patterns of malformation. These disorders include Cornelia de Lange syndrome (CdLS; OMIM 122470). RBS as well as CdLS are considered the prototype of the cohesinopathies, a group of diseases caused by mutation in genes regulating cohesin, a complex considered to be the molecular glue that holds sister chromatids together from S phase until mitosis. CdLS is an autosomal dominant multisystem developmental disorder caused by mutations in NIPBL, SMC1A, SMC3 and PDS5B.[28] Although RBS and CdLS share some clinical traits such as growth and mental retardation, craniofacial and limb malformations and cardiac defects, the pattern of malformations is different. The characteristic facial features in CdLS include synophrys, long eyelashes, depressed nasal root with an uptilted tip of the nose

and anteverted nares, long philtrum, thin upper lip, small widely spaced teeth, small brachycephalic head, and low-set, posteriorly angulated ears. Limb malformations occur in one-third of patients with CdLS in whom upper limb involvement ranges from small hands with single palmar creases and subtle changes in the phalanges and metacarpal bones to severe forms of oligodactyly and truncation of the forearms that primarily involves the ulnar structures. Furthermore, patients with CdLS often present with gastro-oesophageal dysfunction, which has not been reported in RBS.

Despite the lack of general correlation between type and/or location of the mutation and clinical severity, we found evidence that some mutations may correlate with the absence or presence of corneal opacities. Information on this feature was available for 36 patients, 30 of whom had homozygous mutations (online supplementary table 2). Twenty of these homozygous individuals shared their mutation with at least another related or unrelated person, and we found that 18 patients sharing mutations were concordant for the presence or absence of corneal opacities. These include seven with the 750_751insG mutation, six with the 879_880delAG mutation, three with the 505C>T mutation, and two with the 1597_1598insT mutation. Importantly, the six patients with the 879_880delAG mutation come from Turkish, Sudanese and Dutch families which do not share the same origin, as indicated by different haplotypes, suggesting that genetic background may not be relevant for this possible association.[8] Only two homozygous individuals with the same mutation (1131+1G>A; patient 3303 in our cohort and the patient from family 3 in Schüle et al[6]) differed with respect to corneal phenotype. When we analysed phenotypic associations in all cases, including patients with heterozygous mutations, we found that corneal opacity was associated with mental retardation and cardiac abnormalities (table 3). In addition to these associations, it is worth noting that patients from families 5, 6, 7 and 8 who share the c750_751insG mutation did no present with mental retardation, suggesting that some mutations may not affect intellectual capabilities.

Previous studies have shown the expression of ESCO2 in brain, liver, heart, kidney and lung of human embryonic tissues by northern blot.[5] We found that, in human embryos, ESCO2 is expressed in brain, first and third branchial arcs, otocyst, dorsal root ganglia, limb buds, kidney and gonads at CS 14, CS 17 and CS 21 (figure 3). A similar expression pattern is found in the Genepaint database (http://www.genepaint.org) which shows Esco2 expression in brain, gonads, kidney, forelimb, hindlimb and thymus in mouse embryo at E14.5.[29] Although we did not detect ESCO2 expression in the eye or cardiac tissue in human embryos, Esco2 is expressed in these structures in the mouse. Thus, the tissues in which ESCO2 expression was detected appear to correspond to the mature structures that are most severely affected in RBS.

Our studies extend the spectrum of ESCO2 mutations associated with RBS and delineate a characteristic pattern of malformation that distinguishes RBS from other syndromes involving limb and craniofacial malformations. Furthermore, our studies of expression during early human development are important to understand the pleiotropy observed in this syndrome.

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