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Wolcott-Rallison syndrome: pathogenic insights into neonatal diabetes from new mutation and expression studies of EIF2AK3

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Wolcott-Rallison syndrome (OMIM 226980) is a rare autosomal recessive disorder characterised by permanent insulin requiring diabetes developing in the newborn period or early infancy, an early tendency to skeletal fractures, and spondyloepiphyseal dysplasia.¹⁻⁸ The syndrome results from mutations in the gene encoding the eukaryotic translation initiation factor 2- α kinase 3 (*EIF2AK3*, also called *PERK* or *PEK*).⁹ This enzyme phosphorylates EIF2A at Ser51 to regulate the synthesis of unfolded proteins in the endoplasmic reticulum.¹⁰ Targeted disruption of the *Eif2ak3* gene in mice also causes diabetes because of the accumulation of unfolded proteins triggering β cell apoptosis.^{11, 12} Although these murine models have provided significant insight into the pathogenesis of Wolcott-Rallison syndrome, only three human cases have been characterised genetically.^{8, 9} Here, we report genetic analysis of two further cases, and demonstrate new features of the expression pattern of human EIF2AK3 that offer possible explanations for important clinical features of the syndrome that are not apparent in the transgenic mouse models.

METHODS

Primers were designed to amplify all *EIF2AK3* exons and splice site sequences from genomic DNA (table 1). Sequences were amplified by 35 cycles of polymerase chain reaction (PCR) using a proof-reading DNA polymerase in 50 μ l reactions and purified (Qiaquick, Qiagen, Crawley, Sussex, UK). Products were sequenced using the BigDye terminator cycle sequencing kit according to the manufacturer's instructions (Perkin-Elmer, Foster City, California, USA) and an ABI 377 sequencer (Applied Biosystems, city, county, UK). Sequences were compared to the published *EIF2AK3* gene by BLAST analysis (<http://www.ncbi.nlm.nih.gov/BLAST/>). For potential mutations, the PCR and sequencing was repeated to confirm the result. Restriction digest analysis was also used to confirm the mutation in case 2, where the G \rightarrow A substitution destroyed an *Hph*I site.

Optimal conditions were determined for the anti-human EIF2AK3 antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA) by testing the use of several blocking and antigen unmasking techniques. Previously, specimens used in this study, which were obtained with permission of our local ethics committee, have shown positive immunoreactivity to a range of antibodies, indicating satisfactory tissue preparation.

Slides were dewaxed, rehydrated, and washed in phosphate buffered saline (PBS). Sections were pretreated with 3% (vol/vol) hydrogen peroxide in PBS to quench endogenous peroxidase before antigen retrieval by boiling in 0.3 M sodium citrate for 10 minutes and incubation with primary antibody (1:50 dilution) overnight at 4°C. Sections were washed in PBS and incubated with biotinylated anti-goat secondary antibody for two hours at 4°C (1:300; Vector

Laboratories, Burlingame, California, USA). Further washing was followed by incubation for one hour at room temperature with streptavidin conjugated horseradish peroxidase (1:200; Vector Laboratories) and the colour reaction developed over three minutes with diaminobenzidine (10 mg/ml) containing 0.1% hydrogen peroxide. Dual immunohistochemistry was done sequentially with antibodies to islet hormones (all 1:100; Zymed Laboratories, San Francisco, California, USA) using alkaline phosphatase labelled secondary antibodies (1:500) and the Vector red staining kit according to the manufacturer's instructions (Vector Laboratories). Sections were counterstained with toluidine blue and viewed with a Zeiss Axioplan2 imaging system.

RESULTS AND DISCUSSION

Case reports and mutational analyses

Case 1

The clinical history has been detailed previously, and causative mutation of the *PAX4* gene (required for β cell development) was excluded.⁶ This individual, born prematurely at 28 weeks' gestation, was the offspring of consanguineous Saudi parents. The diagnosis of diabetes was made on the fourth day of postnatal life, although insulin was withdrawn on day 28 until the child re-presented with

Key points

- Wolcott-Rallison syndrome, a rare cause of permanent neonatal diabetes and spondyloepiphyseal dysplasia, results from mutations in the gene encoding EIF2AK3.
- We have identified two novel mutations in the *EIF2AK3* gene from unrelated cases of this syndrome, including the first example of splice site mutation as the pathogenic mechanism.
- Additional phenotypic features included a predilection to severe unexplained hypoglycaemic episodes suggestive of hepatic impairment, and to renal failure. These features are not seen in *Eif2ak3* knockout mice.
- Immunohistochemical analysis of human adult and fetal tissues showed that EIF2AK3 was widely expressed in the epithelial cells of the early fetal pancreas, and was present in adult β cells and exocrine tissue. It was also expressed in developing bone, kidney, and adult liver, consistent with the extended phenotype of Wolcott-Rallison syndrome.
- These results both broaden the previous molecular genetic description of this syndrome and provide new information relevant to the pathogenesis of those clinical features in affected patients that are not manifested by transgenic mouse models.

Table 1 Primers used to amplify the *EIF2AK3* gene

Exon	Forward primer	Reverse primer	Product size (bp)	Annealing temperature (°C)
1	Gagaggcaggcgcagtg	cgcgcgtaacaagttgc	403	58.9
2	Tgagcatgtgggataagtc	tgccctaaggggacacaaac	333	60
3	Tcaggatcaagactccagtc	tgacaacctcaggggaaat	448	59.7
4	Ggagltggtaataactgatgc	ccaacagcaacattatctgaa	328	62.5
5	Cccctcttggcataaac	ctgggagaggagaaccgta	449	58
6	Tacttgggctctcagttg	ggcactctgaagtaggaagg	374	58
7	Cctccctgtttgttgaa	gggcaagacagtcaggait	389	60
8	Ctgggccattgttaact	tgaaattgtctccaagatg	384	55.6
9	Tagttaagacgggcctatt	caagagtagcttgggtggag	396	56
10	Aagactggaggatagcagt	agatcttaggtcattctctttg	371	58.1
11	Tgaactgatttcacattaccac	aattggcagcacttgaacc	340	58
12	Gcctcaggggtgttact	cattgtaatcacacaagcaaa	384	56
13	Acagaggtgcagttcaggt	cacaatggttccaatatgc	357	58
13	Aaggtaacaggagagaacct	acctctgctcagatgctt	555	58
14	Catgcaaccactgtact	ctggaacactactccagttt	348	56
15	Cttgggattcaataatgct	ccaatctgctgtaataagaa	288	57.5
16	Tgtggaactgtgggatgtg	tgcaaggacccttacgtt	356	56
17	Tttgccagactgattta	ttcaagctgcaattttgg	367	58.2

Amplification of exon 1 required the addition of 5% dimethyl sulphoxide to the reaction mixture.

diabetic ketoacidosis at the age of four months. Management was complicated by recurrent hypoglycaemia until death at two years from severe diabetic ketoacidosis and infection. Skeletal findings were consistent with spondyloepiphyseal dysplasia and included bilateral femoral fractures.⁶ Direct sequencing of *EIF2AK3* revealed a homozygous deletion of four nucleotides (1563delGAAA) at the site of a GAAA 4 base pair (bp) direct repeat in exon 9. This generates an immediate premature termination codon at amino acid 523 (fig 1). DNA from both the parents and from the proband's unaffected sister was sequenced and heterozygous deletions confirmed in all three individuals. No other alterations were observed in

the exons or immediately adjacent intronic regions in the affected individual or in either parent.

Case 2

Clinical features of the second case up to the age of four and a half years were described previously and included spondyloepiphyseal dysplasia and generalised osteoporosis.⁴ This child, again the offspring of consanguineous Saudi parents, was diagnosed at two months with diabetes requiring insulin therapy, but subsequently died of renal failure. The clinical course was marked by severe intellectual impairment and frequent unpredictable hypoglycaemic episodes. A brother,

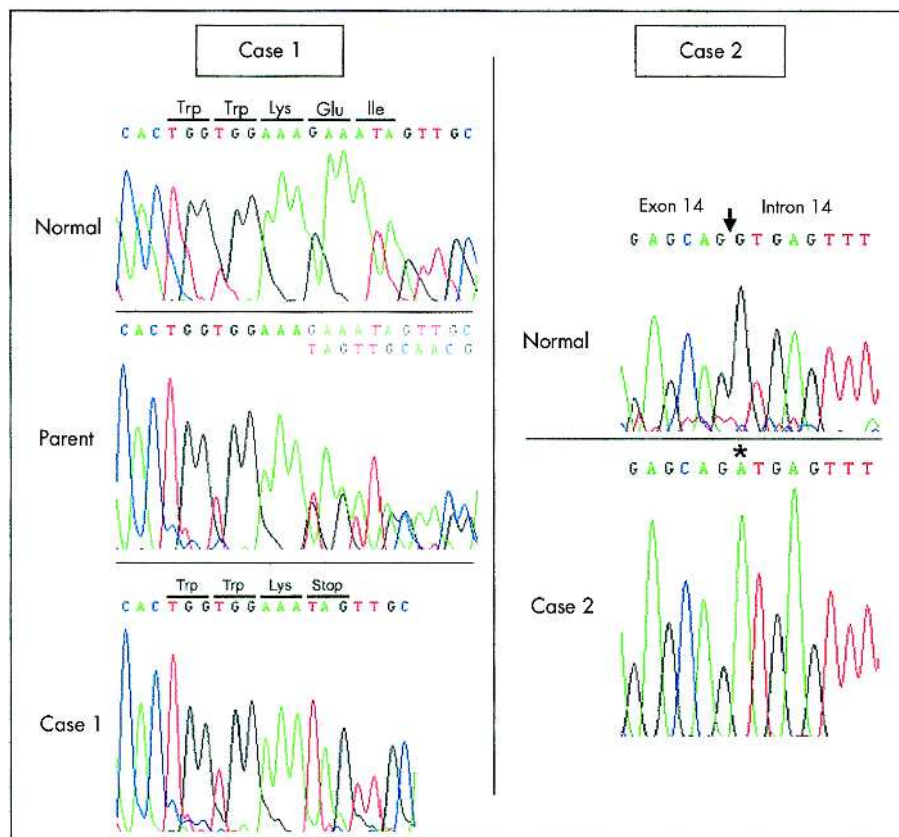


Figure 1 Identification of Wolcott-Rallison syndrome mutations in *EIF2AK3*. Case 1 (left panel): sequences shown for normal DNA, heterozygous parents, and case 1. Deletion of GAAA within one allele of parental DNA and both alleles of case 1 causes a frameshift in exon 9 and premature stop codon at amino acid 523. Case 2 (right panel): substitution at +1 position of intron 14 (IVS14+1G>A; asterisk in case 2) disrupts the donor splice site (arrow above normal DNA). G and T are present with 100% frequency at the +1 and +2 positions, respectively.¹³

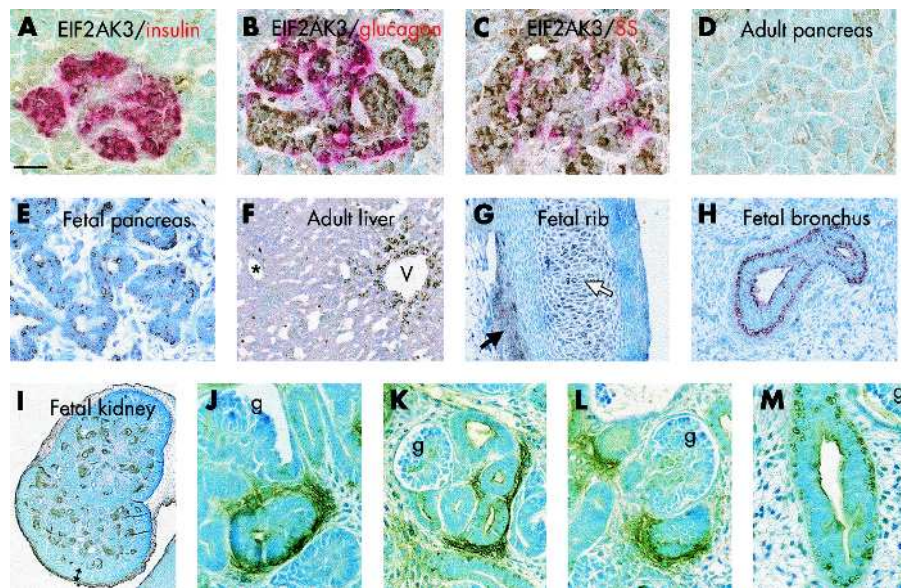


Figure 2 Bright field localisation of EIF2AK3 protein in human fetal and adult tissues. Panels A to C: sections of adult pancreatic islet, counterstained with toluidine blue. Immunohistochemistry shows brown diaminobenzidine staining for EIF2AK3 and red alkaline phosphatase staining for insulin (A), glucagon (B), and somatostatin (SS) (C). Note the overlapping red and brown staining in β cells (A) compared with the largely discrete profiles in (B) and (C). (D) Section of adult pancreatic exocrine tissue. (E) Nests of fetal pancreatic epithelial cells within mesenchymal stroma at 8 wpc. (F) Section of adult liver; *biliary ductule; v, hepatic venule. (G) Longitudinal section of fetal rib at 8.5 wpc. Black arrow indicates perichondrial layer; white arrow points to central cartilaginous matrix. (H) Fetal bronchus at 8.5 wpc. Panels I to M: fetal kidney at 8.5 wpc. (I) Note paucity of staining in outer cortex (double headed arrow). (J) to (M): Higher magnification views of (I) showing three examples of dense peritubular stromal staining, closely associated with glomeruli (g) ((J) to (L)) and tubular staining (M). Note the similarity of intracellular staining between the fetal epithelial structures of (E), (H), and (M). Size bar represents 150 μ m ((A) to (E), (G), (H), (J) to (M)), 250 μ m (F), and 750 μ m (I). Wpc, weeks postconception.

now nine years old, also has insulin dependent diabetes. Diagnosed two weeks after birth, his management is complicated by frequent hypoglycaemia. Two additional siblings are unaffected. No DNA was available from these latter three siblings, or from either parent. Direct sequencing of genomic DNA from the deceased child revealed no *EIF2AK3* coding sequence alterations. However, a homozygous G \rightarrow A substitution was observed at position +1 of intron 14 (IVS14+1G>A; fig 1). Neural network prediction program analysis (http://www.fruitfly.org/seq_tools/splice.html) shows that this alteration, which was not present in 100 normal chromosomes, abolishes the donor splice site at the exon 14/intron 14 boundary. A G nucleotide is invariant at the +1 position of donor splice sites¹³ and G \rightarrow A substitution at this position, identified here, is the commonest mutation to account for splicing abnormalities. RNA from this patient was not available for analysis. However, inclusion of part or all of intron 14 in the mRNA as a result of loss of this splice donor site would bring a stop codon into frame after 75 nucleotides.

Both of these mutations in *EIF2AK3* are novel. However, as with one of the three previously described mutations,⁹ they are both predicted to result in truncated proteins that lack the critical kinase domain. In the other two published cases, single base pair substitutions that affect the kinase domain were described, in one case altering a highly conserved site⁹ and in the other resulting in impaired in vitro function.⁸

Expression of EIF2AK3 in human tissues

Targeted mutation of the mouse *Eif2ak3* gene results in animals that appear normal at birth but have progressive postnatal β cell failure.^{11 12} However, in humans, EIF2AK3 was previously identified by immunohistochemistry only in somatostatin positive δ cells of the pancreatic islet.¹⁴ To try to resolve this anomaly, we studied fixed sections of human adult pancreas by immunohistochemistry with a polyclonal

antibody to EIF2AK3. In adult pancreas, we found that EIF2AK3 was expressed extensively in the islet, with a predominance in β cells (fig 2, panels A to C). Furthermore, in keeping with the exocrine pancreatic insufficiency reported in a human patient⁷ and in one of the knockout mouse models,¹² weaker EIF2AK3 staining was also apparent within the acinar tissue (fig 2, panel D).

The normal birth weights of infants with Wolcott-Rallison syndrome and the unremarkable pancreatic organogenesis in *Eif2ak3*^{-/-} mice^{11 12} might suggest a minimal role for EIF2AK3 during pancreatic development in utero. However, pancreatic hypoplasia/hypotrophy has characterised several cases of Wolcott-Rallison syndrome, either at diagnosis by ultrasound or at necropsy.^{7 8 15} Also, we find that EIF2AK3 is widely expressed within the human fetal pancreas at eight weeks postconception, at which stage the organ is composed of epithelial progenitor cells before islet or exocrine differentiation¹⁶ (fig 2E). Furthermore, in our first case (case 11 of table 2), diabetes developed only four days after premature birth at 28 weeks' gestation. Taken together, these observations indicate that the human fetal pancreas may not be normal in Wolcott-Rallison syndrome. Consistent with this suggestion, mutation of the *Eif2ak3* enzyme target (Ser51Ala of *Eif2a*) resulted in a 50% diminution of pancreatic insulin content between mouse embryonic days 16.5 and 18.5.¹⁷ EIF2AK3 is only one of four kinases known to phosphorylate EIF2A. It is therefore feasible that the precise phenotype of the Wolcott-Rallison syndrome could depend not only on the severity of the *EIF2AK3* mutation, but also on the co-expression and activity of the other kinases. In both humans and mice, there appears to be an absolute requirement for EIF2AK3 in postnatal pancreatic β cells. In contrast, in the murine liver, *Eif2ak3* function appears dispensable.^{11 12} It is therefore interesting to note the recurrent hypoglycaemic episodes and hepatic dysfunction/enlargement that characterises eight of the 15 described cases of Wolcott-Rallison

Table 2 Reported human cases of Wolcott-Rallison syndrome and transgenic mouse models

Features	Human															Mouse		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Harding Eif2ak3 -/-	Zhang Eif2ak3 -/-	Scheuner Eif2aSer51Ala
Age of onset of DM	6 w	8 w	6 w	4 w	3 w	5 w	10 w	8 w	2 w	12 w	4 d§	30 m	<6 m	<6 m	8 w	2-4 w	~3 w	†Hypoglycaemia
Epiphyseal dysplasia	+	+	+	+		+	+	+	+	+	+	+	+	+	+		+	
Osteoporosis/fractures	+		No	+	No	+	No	+	No	No	+	+			No		+	
Hypoglycaemia	+	+						+	+		+				+	No	No	+
Liver dysfunction	+									+		+			+	No	No	+
Hepatomegaly	+		+	+		+				+								No
Renal abnormalities		+				+	+	+		+								No
Birth weight (kg)						3.25			2.80	3.03	1.10§					Normal	Normal	Normal
Clinical exocrine pancreas deficiency												+			No	+	+	No
Pancreatic hypoplasia/hypotrophy										+		+			+	No	No	No
Cardiac abnormality/murmur	+							+	+	+		+						No
Epilepsy (clinical/EEG)		+						+		+					+	No	No	No
Death		2 y			6 w			11 y	<15 y	4 y	2 y		5 m			¶If untreated	¶If untreated	†

Cases 1-3: cases 1, 2, and 3 (siblings) from Wolcott and Rallison, 1972.¹

Cases 4 and 5: cases 1 and 2 from Goumy *et al*, 1980.²

Cases 6 and 7: cases 1 and 2 (siblings) from Stöss *et al*, 1982.³

Cases 8 and 9: cases 1* and 2 (siblings) from al-Gazali *et al*, 1995.⁴

Case 10: Stewart *et al*, 1996;⁵ Thornton *et al*, 1997.¹⁵ Enlarged kidneys, abnormal shape with minor histological abnormalities.

Case 11:* Bonthron *et al*, 1998.⁶

Case 12: Castelnuovo *et al*, 2000.⁷

Cases 13 and 14: Delépine *et al*, 2000.⁹ Although a diagnosis of Wolcott-Rallison syndrome made in these cases, no clinical description was provided.

Case 15: Bignon-Laubert *et al*, 2001.⁸

Where possible a positive phenotypic comment is made, otherwise a box is left blank. An asterisk indicates the cases sequenced in the present study.

§Premature birth at 28 weeks' gestation.

†The Eif2aSer51Ala mice die of hypoglycaemia in the newborn period, although pancreatic insulin content is significantly reduced.

¶Death from hyperglycaemia if untreated.

d, day; DM, diabetes mellitus; m, month; w, week; y, year.

syndrome (ours^{4,6} and those of others^{3,5,8,15} (table 2)). These clinical data are reminiscent of the fatal hypoglycaemia of the Eif2aSer51Ala mutant mice.¹⁷ In combination, it appears likely that in the human but not the mouse hepatic EIF2AK3 may have an important metabolic role that is not compensated for by other enzymes capable of phosphorylating EIF2A. Certainly, EIF2AK3 is strikingly expressed in the perivenous regions of human adult liver (fig 2F), a major site of endoplasmic reticulum associated glycogenolysis.^{18,19} However, it is intriguing that the fatal hypoglycaemia of the Ser51Ala Eif2a mice was associated more with defective gluconeogenesis,¹⁷ a pathway with greater capacity in periportal hepatocytes. The reason for this discrepancy is unclear.

Despite initially normal serum creatinine and renal ultrasound, our second case (case 8 of table 2) subsequently died during childhood of renal failure.⁴ Four other reports of Wolcott-Rallison syndrome have included significant renal impairment^{1,3} and necropsy findings of non-diabetes related pathology.^{3,5,8,15} No renal abnormality has been described in either Eif2ak3 knockout or Eif2Ser51Ala mouse models.^{11,12,17} However, we observe striking EIF2AK3 expression during human fetal renal development. There is minimal expression detected in the outermost nephrogenic cortex (fig 2, panel I), but robust detection is observed in the renal capsule and inner stromal tissue that surrounds tubules (fig 2, panels I to L). EIF2AK3 is also expressed in the developing tubules (fig 2, panel M), with an intracellular distribution similar to that observed in fetal pancreatic and bronchial epithelial cells (fig 2, panels E and H). Finally, EIF2AK3 is detected in the perichondrial layer and inner cartilaginous matrix of the developing limbs and axial skeleton (fig 2, panel G), consistent with the bony malformations that form part of both the human and the murine mutation phenotypes.¹²

Conclusions

We have described two novel mutations in *EIF2AK3*, both of which reinforce the pathogenic significance of loss of the kinase domain—a finding consistent across all five Wolcott-Rallison syndrome mutations characterised to date.^{8,9} The phenotypic features of these cases and of the knockout mouse models are entirely concordant with the expression profile of EIF2AK3 that we observe in human islets of Langerhans and elsewhere in the pancreas, including the lower levels of detection in exocrine tissue. These expression patterns appear much more in keeping with the known clinical features of Wolcott-Rallison syndrome than do previous results that indicated localisation of EIF2AK3 limited to islet δ cells.¹⁴ Furthermore, the likelihood of interspecies differences in the phosphorylation of EIF2A is suggested by the presence in Wolcott-Rallison syndrome of hypoglycaemic complications, hepatic dysfunction, and renal complications that are not observed in transgenic mice models.

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