Brief Genetics Report

Wolcott-Rallison Syndrome
Clinical, Genetic, and Functional Study of \( \textit{EIF2AK3} \) Mutations and Suggestion of Genetic Heterogeneity

Valérie Seneé,1 Krishna M. Vattem,2 Marc Delépine,3 Lynn A. Rainbow,4 Céline Haton,5 Annick Lecoq,6 Nick J. Shaw,7 Jean-Jacques Robert,8 Raoul Rooman,9 Catherine Diatloff-Zito,5 Jacques L. Michaud,10 Bassan Bin-Abbas,11 Doris Taha,12 Bernard Zabel,13 Piergiorgio Franceschini,14 A. Kemal Topaloglu,15 G. Mark Lathrop,3 Timothy G. Barrett,16 Marc Nicolino,6 Ronald C. Wek,2 and Cécile Julier1

Wolcott-Rallison syndrome (WRS) is a rare autosomal-recessive disorder characterized by the association of permanent neonatal or early-infancy insulin-dependent diabetes, multiple epiphyseal dysplasia and growth retardation, and other variable multisystemic clinical manifestations. Based on genetic studies of two inbred families, we previously identified the gene responsible for this disorder as \( \textit{EIF2AK3} \), the pancreatic eukaryotic initiation factor 2\( \alpha \) (eIF2\( \alpha \)) kinase. Here, we have studied 12 families with WRS, totalling 18 cases. With the exception of one case, all patients carried \( \textit{EIF2AK3} \) mutations resulting in truncated or missense versions of the protein. Exclusion of \( \textit{EIF2AK3} \) mutations in the one patient case was confirmed by both linkage and sequence data. The activities of missense versions of \( \textit{EIF2AK3} \) were characterized in vivo and in vitro and found to have a complete lack of activity in four mutant proteins and residual kinase activity in one. Remarkably, the onset of diabetes was relatively late (30 months) in the patient expressing the partially defective \( \textit{EIF2AK3} \) mutant and in the patient with no \( \textit{EIF2AK3} \) involvement (18 months) compared with other patients (<6 months). The patient with no \( \textit{EIF2AK3} \) involvement did not have any of the other variable clinical manifestations associated with WRS, which supports the idea that the genetic heterogeneity between this variant form of WRS and \( \textit{EIF2AK3} \) WRS correlates with some clinical heterogeneity. \textit{Diabetes} 53:1876–1883, 2004

The identification of susceptibility genes for multifactorial disorders, such as type 1 diabetes, presents many challenges. To date, only two susceptibility loci for type 1 diabetes have been unambiguously identified, \textit{IDDM1} (HLA locus) and \textit{IDDM2} (insulin gene locus). Genetic studies of monogenic forms of diabetes-related disorders can significantly increase our knowledge of \( \beta \)-cell function and may point to potential candidate genes for the common forms of diabetes. Much information has been gained on studying syndromes such as maturity-onset diabetes of the young (1,2), Wolfram syndrome (3), and, more recently, Wolcott-Rallison syndrome (WRS) (4). WRS is a very rare syndrome, with less than 20 cases described in the world literature (4–17). It associates permanent neonatal or early-childhood insulin-dependent diabetes and epiphyseal dysplasia. Other clinical features that show variability between WRS cases include mental retardation, hepatic and kidney dysmorphisms, and other abnormalities such as congenital cataracts, microperforations of the lens, and obesity. Based on genetic studies of two consanguineous families, we previously identified the gene responsible for this disorder as \( \textit{EIF2AK3} \) (or \( \textit{PEK} \)), the pancreatic eukaryotic initiation factor 2\( \alpha \) (eIF2\( \alpha \)) kinase (4). In addition, two independent knockout mice have been produced and studied that show a phenotype remarkably similar to human WRS, with neonatal diabetes, skeletal defects, and small size with delayed growth (18,19). Recent reports have begun to identify mutations in \( \textit{EIF2AK3} \) genes linked with WRS (4,16,17). Together, these
studies definitively establish that EIF2AK3 mutations are responsible for WRS. However, the variability of the clinical manifestations observed in WRS remains unexplained. In the present study, we have assembled and studied a large collection of cases and families with WRS in order to extend the clinical and genetic investigation of this syndrome and to explore the determinism of its clinical variability.

A total of 12 families (18 patients with WRS) were studied, and detailed clinical information was obtained on these patients. This is the largest collection of WRS patients studied to date. The summary description of these patients is shown in Table 1. Patients were from various population origins, and, in most cases, their parents were known to be consanguineous (families WRS1, -2, -3, -4, -5, -6, -7, -8, -10, and -12) or likely to be since they lived in the same village (WRS9). There was no evidence of consanguinity in one case (WRS11). The age at onset was generally very young: 15 had an onset between the ages of 5 weeks and 6 months, but 2 had a significantly older age at onset, at 18 months (WRS12-1) and 30 months (WRS10-1). Excluding these two outliers, the mean age at onset was 3 months. Most of these cases were still alive (aged between 3 months and 35 years), and some had died (aged between 3 months and 35 years). Diabetes required insulin therapy from the onset of the disease. With one possible exception, there was no evidence of autoantibodies (islet cell antibody, insulin autoantibody, IA-2 antigen, and GADA) in the patients at the onset of disease when this was tested. Slightly elevated values were found for patient WRS12-1 at disease onset (GADA: 2.3 IU) but within normal range (0.94 IU) 3 months later. In addition to the multiple epiphyseal dysplasia common to all patients, there were various degrees of osteopenia, ranging from undetected or mild (possibly detected by bone densitometry) to severe, with multiple fractures. The mental and psychomotor development was assessed based on the sitting/walking age, speech and communication, and school performance. There was slight to severe mental retardation or developmental delay in the majority of the patients, but some were within the normal range (WRS9-1, 10-1, 11-1, and 12-1). Epilepsy was not reported in any of these patients, in contrast with other reports (5). Recurrent episodes of hepatitis occurred in the majority of the patients. These were major events, requiring hospitalization, often accompanied by acute renal failure and sometimes resulting in death. Hepatic and renal functions returned to normal for those surviving these episodes. In all cases where it was explored, no causative viral infection could be identified. In addition, progressive chronic renal insufficiency was noted in three patients, WRS8-1, WRS9-1, and WRS9-2, who were also the ones who lived to older ages (between 11 and 35 years). Four patients showed signs of exocrine pancreas dysfunction, WRS2-1, WRS4-1, WRS5-1, and WRS10-1, with pancreatic hypotrophy in WRS10-1 (12) and fibrosis infiltrations in pancreas...
biopsy in WRS2-2 (11). Central hypothyroidism was noted in four patients (WRS1-1, WRS4-1, WRS7-1, and WRS7-2) (14,15), but thyroid function was normal in the others. Neutropenia was reported in nine patients (WRS2-1, WRS2-2, WRS3-1, WRS4-1, WRS5-1, WRS6-1, WRS10-1, and WRS11-1), who also tended to suffer from frequent infections (bacterial, viral, and fungal). The phenotype of the parents and heterozygous siblings was not remarkable; in particular, none had diabetes.

Sequencing of the coding regions of *EIF2AK3* was performed in all the WRS cases and in available parents and siblings. *EIF2AK3* mutations were identified in 11 of the 12 families, in the homozygous state in the patients of 10 of them, and as a compound heterozygote in WRS11-1, whose parents were not consanguineous. In this last family, segregation of the alleles was consistent with linkage to *EIF2AK3*, since an unaffected sibling received different parental *EIF2AK3* alleles than the affected case, based on microsatellite genotyping and sequence analysis (data not shown). None of these 12 mutations were found in a control population of 95 Caucasian individuals (4).

The nature, position, and consequences of these mutations are shown in Table 2 and displayed in Fig. 1, together with other previously reported mutations (16,17) and polymorphisms (4). Two novel mutations and a previously reported one were nonsense mutations, resulting in truncated proteins 162, 520, and 522 amino acid residues in length. Five novel mutations were frameshift mutations resulting in truncated proteins, with length varying between 344 and 1,024 correct amino acid residues compared with 1,115 for the full-length EIF2AK3. One *EIF2AK3* mutation was a splice-site mutation, resulting in a truncated protein with 994 correct residues. The other five novel mutations, as well as a previously reported one, were missense mutations, all located within the catalytic domain of the protein (Fig. 1). In contrast, the four frequent amino acid variants (polymorphisms) that we have identified in the Caucasian population (4) are located at various positions of the protein, but all outside of the kinase domain: one in the predicted signal peptide, two in the regulatory domain, and one in the insert region in the kinase domain.

All of the *EIF2AK3* mutations that resulted in truncated proteins are missing all or part of the kinase domain, which would be expected to lead to a complete loss of function (Fig. 1). To evaluate the functional consequence of the missense mutations, we measured the activity of *EIF2AK3* mutant proteins using the well-characterized yeast translation assay (20). The *EIF2AK3* catalytic domain was fused to an NH2-terminal glutathione S-transferase (GST) tag and expressed in yeast strain H1894, which was deleted for its sole endogenous eIF2α kinase. Expression of the wild-type version of *EIF2AK3* using a galactose inducible promoter blocked growth of the H1894-derived cells in the inducing SGAL medium (Fig. 2A). No growth defect was observed in the glucose medium (SD), in which *EIF2AK3* protein is poorly expressed at serine-51 (Fig. 2B). No growth defect was observed in the glucose medium (SD), in which *EIF2AK3* protein is poorly expressed. This growth deficiency was due to hyperphosphorylation of eIF2α, as measured by immunoblotting using a polyclonal antibody that recognizes eIF2α phosphorylated at serine-51 (Fig. 2B). By contrast, expression of a mutant version of *EIF2AK3*K621M altered in the invariant lysine required for kinase catalytic activity did not induce eIF2α phosphorylation or elicit a growth defect in the SGAL medium.

We next measured the activities of the newly identified WRS missense mutations using the yeast translation system. Yeast strains expressing each of the five *EIF2AK3* mutants...
showed efficient growth in the SGAL-inducing medium and no detectable eIF2α phosphorylation as measured by immunoblot (Fig. 2A and B). Levels of the mutant EIF2AK3 proteins in yeast were in fact higher than the wild-type counterpart, indicating that the residue substitutions did not impact their expression or stability. It is noted that the mutant EIF2AK3 proteins migrated faster than the wild-type version in the SDS-PAGE analysis preceding the immunoblot, due to autophosphorylation of EIF2AK3 that accompanies its activation (21). Phosphorylation in this assay (Fig. 2C). These results indicate that four of the missense mutations (R587Q, L645P, W898C, and L1057P) result in a complete loss of EIF2AK3 function in vivo and in vitro, while one (N655K) retains some kinase activity.

No homozygous or heterozygous mutations were detected in patient WRS12-1 or in the EIF2AK3 exons and 3′ untranslated and promoter regions. Genotyping of five adjacent microsatellite polymorphisms encompassing the EIF2AK3 gene excluded linkage to WRS in this family (Fig. 3). In addition, the sequence of EIF2AK3 exons in this family showed that the patient was heterozygous at four genetic variants within this gene (data not shown). Although it is formally possible that this patient has inherited independent EIF2AK3 mutations from each parental.

### TABLE 2
Nature of mutation

<table>
<thead>
<tr>
<th>Kidney dysfunction (chronic)</th>
<th>Kidney dysfunction (acute)</th>
<th>Neutropenia</th>
<th>Frequent infectious episodes</th>
<th>Hypothyroidism</th>
<th>Exocrine pancreas dysfunction</th>
<th>Autoantibodies</th>
<th>EIF2AK3 mutation</th>
<th>Nature of mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>WRS1-1</td>
<td>WRS1-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+ W163STOP</td>
<td></td>
</tr>
<tr>
<td>WRS2-1,2,3</td>
<td>WRS2-1,2,3</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+ fs345/STOP345</td>
<td></td>
</tr>
<tr>
<td>WRS3-1*</td>
<td>WRS3-1*</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>fs345/STOP345</td>
<td></td>
</tr>
<tr>
<td>WRS4-1</td>
<td>WRS4-1</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+ W521STOP</td>
<td></td>
</tr>
<tr>
<td>WRS5-1</td>
<td>WRS5-1</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+ W898C, L1057P</td>
<td></td>
</tr>
<tr>
<td>WRS6-1</td>
<td>WRS6-1</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>WRS7-1,2</td>
<td>WRS7-1,2</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>WRS8-1,2</td>
<td>WRS8-1,2</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>WRS9-1,2</td>
<td>WRS9-1,2</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>WRS10-1</td>
<td>WRS10-1</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>WRS11-1†</td>
<td>WRS11-1†</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>WRS11-1†</td>
<td>WRS11-1†</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td></td>
</tr>
</tbody>
</table>

*Patient homozygote for two mutations; †patient compound heterozygote for two mutations. fs, frameshift mutation, starting at the position shown.
ent, which would both be located in distant regions of the gene or in introns, this is highly unlikely given the fact that the 15 EIF2AK3 mutations identified so far in WRS affect the amino acid sequence. Together, these observations support the idea that the WRS in this patient is not caused by EIF2AK3 mutations. Since his parents are consanguineous, his syndrome is likely to result from a recessive mutation in another single gene, although we cannot exclude that the observed diabetes and bone dysplasia occurred by coincidence in this patient. In the first hypothesis, the responsible gene may be one of the alternative eIF2α kinase, another gene involved in this pathway, or interacting with EIF2AK3. Using microsatellite markers encompassing GCN2, HRI, and PKR genes, we could exclude linkage of WRS in WRS12 family to the regions of these three alternative eIF2α kinases (data not shown).

Other than the bone dysplasia and the presence of neonatal or early-infancy onset of insulin-dependent diabetes, present in all patients, there was marked variability in the other features frequently associated with WRS (Tables 1 and 2). We questioned whether this variability may be explained in part by the nature of the gene (EIF2AK3 or not) or the type and location of the EIF2AK3 mutation. Most patients had a very early age at onset (<6 months), but two of them (WRS10-1 and 12-1) had a relatively old age at onset (30 and 18 months, respectively). Remarkably, WRS10-1 carried the N655K mutation, which was the only one with residual kinase activity, while all of the other mutant versions of EIF2AK3 either resulted in truncated proteins or had no catalytic activity; and WRS12-1 was the only patient with no implication of the EIF2AK3 gene. In addition to the late age at onset, WRS12-1 was the only patient lacking all of the other frequent complications observed in WRS patients. Borderline positivity for GADA autoantibodies was also found in this patient at the onset of the disease, which did not persist after 3 months. HLA typing showed that this patient was DR3 positive and DR4 negative (not shown), which does not allow us to make any conclusions on the autoimmune nature of diabetes in this patient. Based on these observations, the WRS12-1 patient appears to have a variant form of WRS, with genetic and slight clinical heterogeneity compared with EIF2AK3-caused WRS.

Apart from age at onset, there was poor concordance overall between the nature of the EIF2AK3 mutation and the variable features of the disease, suggesting that other genetic factors (modifier genes) or environmental factors may be involved. In particular, some affected siblings from the same family were discordant for mental retardation (family WRS9), the presence of acute or chronic hepatic or kidney dysfunction (families WRS2 and WRS8), and exocrine pancreas dysfunction (family WRS2). The patients who had chronic kidney dysfunction were the older living patients (age >11 years), suggesting that this feature may be a long-term complication of this syndrome. However, the absence of acute episodes or chronic conditions may not be a significant phenotype in young children, who may develop these at a later age. Possible familial aggregation was found for neutropenia/frequent infections, osteopenia, and hypothyroidism; however, it is difficult to conclude whether these complications are related to specific mutations because of the limited number of observations.
Overall, our data suggest that factors unrelated to \textit{EIF2AK3} gene contribute to a large extent to the clinical variability of WRS. Variation in environmental conditions that lead to endoplasmic reticulum stress or genetic variation in other genes involved in the response to endoplasmic reticulum stress are likely to modulate the severity and clinical characteristics of the disease. Such an effect of environmental stress factors on disease severity and progression has been shown in a rare recessive neurological disorder, leukoencephalothy with vanishing white matter, which is caused by mutations in subunits of the eIF2B translation initiation factor (22,23).

Parents and heterozygous siblings of WRS patients from this study had no remarkable features; in particular none had type 1 or type 2 diabetes or bone disorder. This suggests that \textit{EIF2AK3} may not play a major role in the susceptibility to frequent forms of diabetes, at least as a single gene, despite our observation of linkage of the \textit{EIF2AK3} region to type 1 diabetes in the Scandinavian population (24).

Parents and heterozygous siblings of WRS patients from this study had no remarkable features; in particular none had type 1 or type 2 diabetes or bone disorder. This suggests that \textit{EIF2AK3} may not play a major role in the susceptibility to frequent forms of diabetes, at least as a single gene, despite our observation of linkage of the \textit{EIF2AK3} region to type 1 diabetes in the Scandinavian population (24).

Our studies suggest the existence of a variant form of WRS, which does not present any of the complications frequently observed in this syndrome and may be associated with an older age at onset. In addition, the only patient who carried a homozygous \textit{EIF2AK3} mutation with a slight residual activity in our functional studies was found to have a significantly delayed age at onset. Therefore, we propose to extend the age-at-onset definition in WRS to “early-infancy onset” instead of the generally used “neonatal” diabetes. Based on our observations, a diagnosis of WRS should be considered in patients presenting with insulin-dependent diabetes starting at older ages, in case of an association with epiphyseal dysplasia, and the \textit{EIF2AK3} gene screened for mutations in these patients. In addition to these genetic heterogeneity factors, part of the variability of WRS is likely to be due to additional factors, such as modifier genes or factors related to the environment or patient’s management, or to the natural progression of the disease.

RESEARCH DESIGN AND METHODS

A total of 12 families (18 patients with WRS) were studied, including the two original families that we previously reported (4), and other previously reported cases from Germany (6), Mauritania/France (12), and Saudi Arabia (13–15). Six other families from Quebec, Iran/Belgium, Pakistan/U.K., Slovakia, Italy, and Turkey have not been previously reported. All patients and families gave their informed consent for genetic studies, which were approved by local institutional committees. WRS diagnosis was made on the basis of the minimum association of neonatal or early-childhood insulin-dependent diabetes and multiple epiphyseal dysplasia, as described (5). A detailed question-
naire was provided to all the clinicians in order to collect additional clinical and familial information. A description of these cases is provided in Table 1.

**Mutation screening.** DNA was extracted from peripheral blood collected on EDTA and EIF2AK3 mutation screening was performed on genomic DNA on all the coding regions of the gene in the cases and their parents (when available), as previously described (4). Alternatively, RNA was extracted from peripheral blood and sequencing performed on the cDNA (4). Additional sequencing was performed in one patient and his parents (family WRS12) to cover the 3’ untranslated region of the gene, as previously described (4), and exon 1 and 1–1,500 bp of sequence 5’ of the starting ATG of the gene, using three sets of overlapping primers: 5p3f/373–377, 5p2f/5p2r, and 5p1f/5p1r (template sizes: 962, 554, and 574 bp, respectively), with 5p2f: 5’-GTCAGAATTCGCCCCATGTAGT-3’, 73–377R: 5’-CGCCGCTAAAGAAATTTG-3’, 5p1f: 5’-AGTTCAGGCACTGCTT-3’, and 3p1r: 5’-GGGCAGATGGAGGAGTACT3’. Sequencing reactions were performed using the amplification primers and an additional primer in the case of 5p3f/373R: 5’-CGAGATAGGCTGTCACTGAGG-CC–3’.

**Microsatellite genotyping.** Microsatellite genotyping was performed using fluorescence-labeled primers on an ABI3700 sequencer, using standard methods. For assessing linkage to the EIF2AK3 region, the following microsatellite marker were used: D2S131, D2S2216, D2S2181, D2S113, and D2S2175.

**Analysis of EIF2AK3 activity.** Functional assays for EIF2AK3 were performed using the yeast strain H1894 (MATa ura3-52 leu2-3, -112 gcn5Δ trpl1Δ63), which is deleted for its sole endogenous eIF2α kinase. The EIF2AK3 gene was inserted downstream of a galactose-inducible promoter in the URA3 gene was inserted downstream of a galactose-inducible promoter in the URA3 gene, and total eIF2α protein levels were measured by immunoblot analysis. Phosphorylated eIF2α was visualized using a fluorescently labeled antibody that recognizes total levels of the GST-tagged EIF2AK3 or an antibody specifically recognizing phosphorylated eIF2α at serine-51 (Biosource International), and total eIF2α protein levels were measured by immunoblot analysis. Phosphorylated eIF2α was visualized using an antibody that specifically recognizes eIF2α phosphorylated at serine-51 (Biosource International), and total eIF2α protein levels were measured by using polyclonal antibody prepared against total yeast eIF2α. The eIF2α-antibody complex was visualized by using horseradish peroxidase–labeled anti-rabbit secondary antibody and chemiluminescent substrate. To measure the steady-state levels of wild-type and mutant versions of EIF2AK3 in yeast, cells were inoculated into SGAL medium at A600 = 0.25 and incubated with constant shaking at 30°C for 20 h. Cells were collected, broken with glass beads, and lysates were clarified by centrifugation as described (20). The GST-tagged EIF2AK3 proteins were purified from 50 μl yeast lysate by using glutathione sepharose. Proteins associated with the sepharose were removed by boiling in SDS sample buffer and separated by electrophoresis in a 10% SDS polyacrylamide gel. Proteins were transferred to nitrocellulose filters, and immunoblot analysis was performed with an antibody that recognizes total levels of the GST-tagged EIF2AK3 or an antibody specific for EIF2AK3 phosphorylated at serine-51 (Biosource International). Reaction mixtures were incubated at 30°C for 4 min, a time point that was found to be the linear range of the assay.

**ACKNOWLEDGMENTS**

This work was supported in part by a grant from the Juvenile Diabetes Research Foundation, INSERM, and Fondation pour la Recherche Médicale (FRM) (to C.J.) and National Institutes of Health grant GM643540 (to R.C.W.). V.S. was a recipient of an FRM postdoctoral fellowship.

We thank Drs. M. Ovickova, M. Korada, and S. Iyer for providing clinical information and blood samples on some WRS patients. We thank the Hospices Civils de Lyon for their support.

**REFERENCES**

