REPORT

Biallelic Mutations of Methionyl-tRNA Synthetase Cause a Specific Type of Pulmonary Alveolar Proteinosis Prevalent on Réunion Island

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Methionyl-tRNA synthetase (MARS) catalyzes the ligation of methionine to tRNA and is critical for protein biosynthesis. We identified biallelic missense mutations in *MARS* in a specific form of pediatric pulmonary alveolar proteinosis (PAP), a severe lung disorder that is prevalent on the island of Réunion and the molecular basis of which is unresolved. Mutations were found in 26 individuals from Réunion and nearby islands and in two families from other countries. Functional consequences of the mutated alleles were assessed by growth of wild-type and mutant strains and methionine-incorporation assays in yeast. Enzyme activity was attenuated in a liquid medium without methionine but could be restored by methionine supplementation. In summary, identification of a founder mutation in *MARS* led to the molecular definition of a specific type of PAP and will enable carrier screening in the affected community and possibly open new treatment opportunities.

Pulmonary alveolar proteinosis (PAP) is characterized by an accumulation of lipoproteins in the pulmonary alveoli; this accumulation leads to restrictive lung disease and respiratory failure.[1–3] PAP is either acquired or inherited in an autosomal-recessive mode. The acquired form (MIM: 610910) affects adults and is attributed to granulocyte-macrophage colony-stimulating factor (GM-CSF) autoantibodies.[4,5] Inherited PAP is usually diagnosed in early childhood. So far, rare mutations in CSF receptor genes *CSF2RA* (MIM: 306250) and *CSF2RB* (MIM: 138981) have been reported as a cause of inherited forms (MIM: 300770, 614370). [6–8] A specific, severe childhood form of PAP is prevalent on Réunion Island, where the incidence is at least 1 in 10,000 newborns.[1,2] Mutations in *CSF2RA* and *CSF2RB* have been excluded previously.[1] Since 1970, approximately 34 children have been diagnosed and treated. If a founder mutation is assumed, the most recent common ancestor of these children can be traced back to the early 18th century.[1] The main symptom is respiratory insufficiency, often leading to death in childhood or adolescence as a result of lung fibrosis despite supportive treatment, including regular whole-lung lavages (Table S1). In addition to lung fibrosis, non-life-threatening liver involvement might be present, as indicated by elevated enzymes, steatosis, fibrosis, or cirrhosis. We investigated 26 DNA samples from individuals who were from Réunion or the nearby islands of Comoros and Madagascar and who were affected with unexplained PAP, and we performed homozygosity mapping and exome and whole-genome sequencing to identify the genetic basis of this disease (Table S2). In addition, we analyzed DNA from a Tunisian sibling pair and from an individual who has sporadic PAP and is living in Paris.[9] Written informed consent was obtained from all study participants. The study was approved by the Comité de Protection des Personnes Île de France II ethical review board.

We performed SNP-array genotyping in 14 affected individuals from the Réunion and Comoros islands by using HumanOmni2.5-4 v.1 and CNV370-Duo v.1 SNP arrays (Illumina); this enabled us to map a homozygous region to chromosome 12q13.3 in all investigated individuals. This region comprised 530 kb between markers rs703817 and rs2277324 (Figure S1) and contained 20 genes. Results from additional SNP genotyping in the two affected siblings from Tunisia were compatible with these findings. Both siblings were homozygous within the critical region on 12q13.3; however, they carried a haplotype different from that found in all affected individuals from Réunion.

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http://dx.doi.org/10.1016/j.ajhg.2015.03.010.

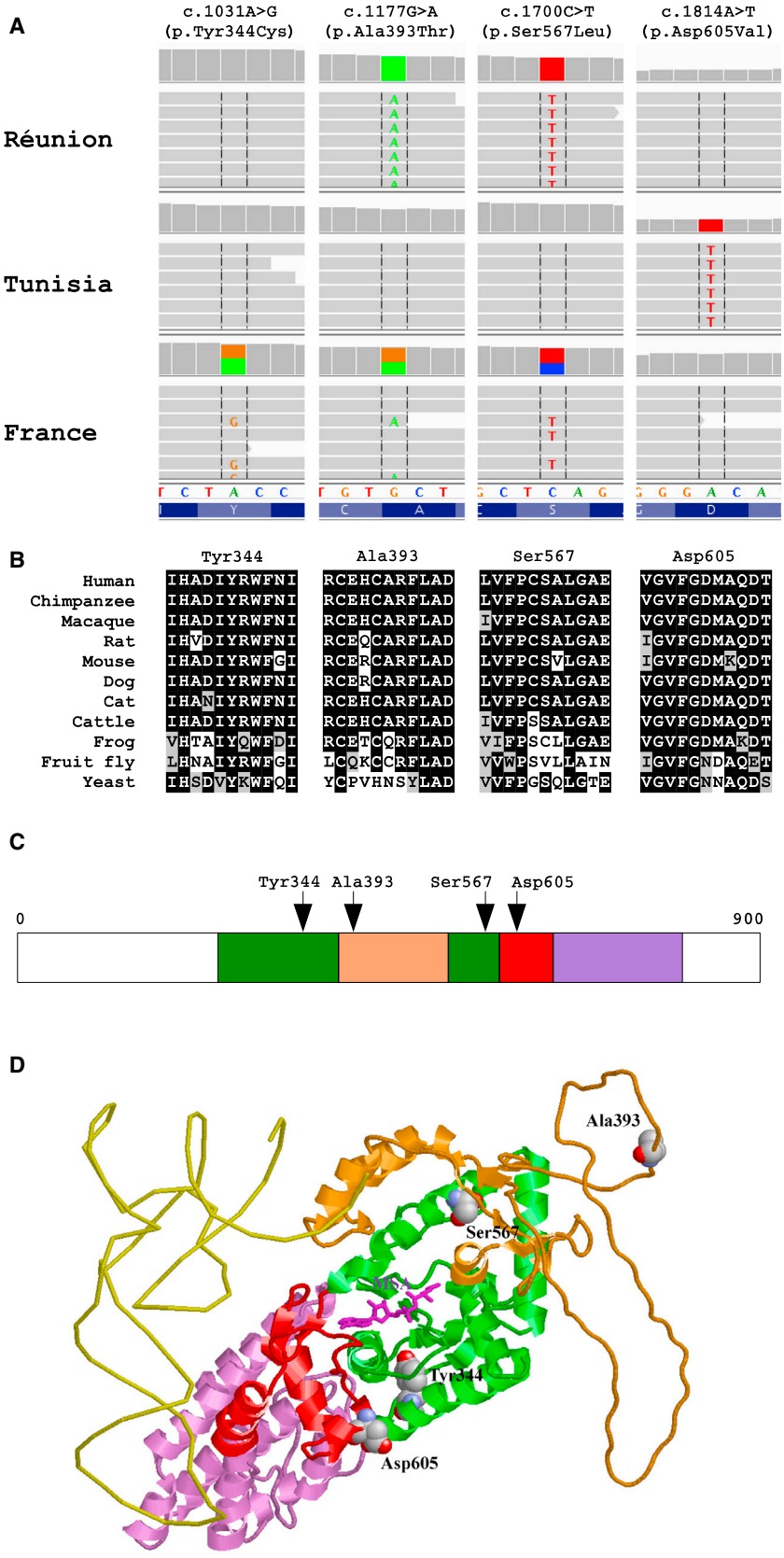

Figure 1. ***MARS*** **Variants in PAP**
(A) Sequencing reads showing the different biallelic variants identified in individuals from Réunion, Tunisia, and France.
(B) Amino acid conservation across MARS orthologs.
(C) Scheme of the domain structure of MARS with the location of the variants.
(D) Predicted tertiary structure. MARS contains a nucleotide-binding (Rossmann) fold (green); a region called the connective polypeptide, which contains the zinc-binding sites (orange); the stem-contact fold domain (red); and the α-helix bundle domain that forms the anticodon-binding site (violet). The positions of the variants are indicated relative to the reference sequence (GenBank: NM_004990.3). The structure of human MARS was predicted by homology modeling based on the *Aquifex aeolicus* structure of MARS complexed with methionyl sulfamoyl adenosine (MSA) and the elongator $tRNA^{Met}$ (PDB: 2CT8)[10] as templates. The model was constructed with the SWISS-MODEL automated protein-structure homology-modeling server. The predicted structure was superimposed with MSA and $tRNA^{Met}$ with SPDBviewer and was visualized with Rasmol.

We performed exome sequencing on two individuals from Réunion and one of the Tunisian siblings to identify possible disease-causing variants. Sequencing was performed via 100-bp paired-end reads on HiSeq2500 systems (Illumina). We generated, on average, 11.3 Gb of sequence, resulting in an average depth of coverage of 135 and in 94% of the target regions' being covered at least 20 times. Single-nucleotide variants (SNVs) and small insertions and deletions were called with SAMtools and Pindel and filtered so that only those variants with a minor-allele frequency (MAF) of less than 1% remained. As controls for filtering, we used 4,000 in-house exomes from individuals with unrelated diseases (Figure S2), the 1000 Genomes Project data (n = 1,700), and the Exome Aggregation Consortium (ExAC) Browser dataset (n = 60,706).

Rare variants common to all three affected individuals were only detected in methionyl-tRNA synthetase (*MARS* [MIM: 156560]), one of the 20 genes in the critical region. Three additional missense variants in the critical region had an allele frequency of at least 0.21 (Table S3). The individuals from Réunion, Comoros, and Madagascar islands carried two homozygous missense variants (GenBank: NM_004990.3): c.1177G>A (p.Ala393Thr; rs141340466) and c.1700C>T (p.Ser567Leu; rs143592405), in exons 10 and 14, respectively. The sibling pair from Tunisia carried

Table 1. Variants Identified in *MARS*

Origin of Individuals	Zygosity	Genome	cDNA	Protein	PPH2	SIFT	CADD
Réunion or Comoros	homozygous	chr12: g.57906083C>T	c.1700C>T	p.Ser567Leu	benign	0.68	17.38
		chr12: g.57894189G>A	c.1177G>A	p.Ala393Thr	benign	0.16	17.63
Tunisia	homozygous	chr12: g.57906594A>T	c.1814A>T	p.Asp605Val	probably damaging	0	26.2
France	heterozygous	chr12: g.57906083C>T	c.1700C>T	p.Ser567Leu	benign	0.68	17.38
		chr12: g.57894189G>A	c.1177G>A	p.Ala393Thr	benign	0.16	17.63
	heterozygous	chr12: g.57892346A>G	c.1031A>G	p.Tyr344Cys	probably damaging	0	23.9

The human genome assembly hg19 (CRCh37) and transcript NM_004990.3 were used as reference sequences. SIFT values below 0.05 are predicted to have functional impact. For CADD, phred-like scores (scaled C scores) are listed.

a different homozygous missense variant, c.1814A>T (p.Asp605Val), in exon 15 (Figure 1, Table 1, and Table S2). The results from Sanger and/or exome sequencing in the remaining 12 affected individuals used for homozygosity mapping and in 12 additional affected individuals from Réunion were consistent with the initial findings. A variant, p.Asp605Gly, was present in a heterozygous state in a single sample of East Asian origin out of the approximately 65,000 samples used for filtering. The variants p.Ala393Thr and p.Ser567Leu carried by the individuals from Réunion were found in four exomes of African origin in the ExAC dataset. In addition, the variant p.Ala393Thr was present in 19 of 4,327 exomes of East Asian origin and in a single exome of different origin. The frequency of the variants p.Ala393Thr and p.Ser567Leu in 1,000 control subjects from Réunion was 22 in 2,000 alleles, resulting in a predicted disease frequency of 1 in 8,264, which is consistent with the observed disease frequency on Réunion. Control subjects were randomly chosen from the Réunion DNA bank, which contains DNA from individuals referred to the Centre Hospitalier Universitaire de la Réunion because of various diagnoses. Individuals with lung diseases were excluded.

Searching for additional disease-causing variants, we sequenced an additional affected individual living in Paris. SNP genotyping was consistent with a constellation in which this individual carried two different haplotypes in the critical region and in which one of these haplotypes was identical with the Réunion haplotype. Indeed, we detected the two variants of the Réunion haplotype and an additional heterozygous non-synonymous substitution, c.1031A>G (p.Tyr344Cys), in exon 9 (Figure 1, Table 1); this substitution was present in a heterozygous state in two exomes of European origin in the ExAC dataset. Capillary sequencing in the parents demonstrated a compound-heterozygous state of both alleles. The Réunion allele was inherited from the mother, who turned out to have been born in Réunion, and the other allele came from the father. We further excluded structural variations at the locus by performing whole-genome sequencing in two affected individuals from Réunion. We generated, on average, 115.5 Gb of sequence, resulting in an average depth of coverage of 32 and in 89% of the coding regions' of the RefSeq collection being covered at least 20 times. In addition, this analysis revealed no evidence for a deletion of any of the coding exons in the critical region.

Homozygous or compound-heterozygous occurrence of rare *MARS* variants was only infrequently observed in control individuals. Our in-house exomes contained only one additional exome carrying a homozygous *MARS* missense variant, c.2180G>A (p.Arg727Gln; rs113808165; MAF = 0.55%), and one compound-heterozygous carrier of the same variant (rs113808165) in combination with the missense variant c.617C>T (p.Pro206Leu; rs138776588; MAF = 0.5%). The two individuals carrying these variants were diagnosed with myocardial infarction and ventricular arrhythmia, respectively. Both missense variants were predicted to be benign by PolyPhen-2 and SIFT. A conservative test comparing the three homozygous or compound-heterozygous variants in the affected individuals with the two homozygous variants found in 4,000 control subjects found the differences to be highly significant (Fisher's exact test: $p < 1.9e{-}8$).

MARS codes for the methionyl-tRNA synthetase, which belongs to the class 1 family of aminoacyl-tRNA synthetases (ARSs). These enzymes play a critical role in protein biosynthesis by charging tRNAs with their cognate amino acids. MARS is a component of a multi-protein complex and catalyzes the ligation of methionine to tRNA molecules. The protein is highly conserved and ubiquitously expressed. Structural prediction of human methionyl-tRNA synthetase (Figure 1 and Figures S3 and S4) showed that Tyr344 and Ser567 lie in the MARS Rossman fold, a domain that contains most of the sites that catalyze both the methionyl adenylation from L-methionine and ATP and the methionylation of the $tRNA^{Met}$. Ala393 lies in a loop in the connective polypeptide, downstream of the first of four conserved CXX[C,D,H] motifs that are involved in the binding of two zinc ions. Asp605 lies in the stem-contact fold domain, which contains both a region that binds to the inside of the L-shaped tRNA and sites that catalyze the methionyl adenylation.[11–13]

Because of the fundamental role of ARSs in cell metabolism, the identified variants most likely result in reduced enzyme activity rather than a complete loss of that activity. Taking advantage of the conservation of MARS between humans and yeast, we assessed enzyme activity of the

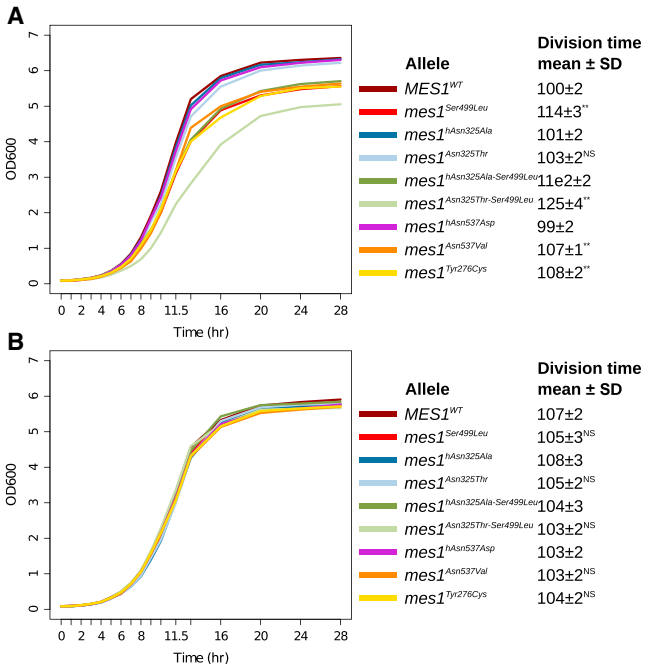

Figure 2. Growth of *MES1* Wild-Type and *mes1* Mutant Strains
Growth (A) without methionine or (B) with 20 μg/ml methionine. Cells were inoculated at the concentration of 0.1 OD_{600}/ml and grown until the stationary phase was reached after 28 hr. At regular intervals, aliquots were used for measurement of cell density by UV-visible spectrophotometry at 600 nm. Sampling times are indicated by x-axis ticks. Tables show division times (minutes) calculated during the exponential phase of growth. Division times are the mean of three independent growth curves. The *S. cerevisiae* strain used in this work was W303-1B (*Matα ade2-1 leu2-3,112 ura3-1 trp1-1 his3-11,15 can1-100*). The *MES1* wild-type allele was cloned in the centromeric vector pFL38[14]. Genomic *MES1* was disrupted in the pFL38*MES1*-transformed W303-1B strain by one-step gene disruption with a KanMX expression cassette.[15] *mes1* mutant and double-mutant alleles were constructed via site-directed mutagenesis through the PCR overlap extension technique with the oligonucleotides listed in Table S4,[16] cloned into vector pFL39[14], and introduced into W303-1B *mes1Δ* pFL38*MES1*. In a second step, strains devoid of pFL38$MES1^{WT}$ and containing the pFL39-borne $MES1^{WT}$ or *mes1* mutant alleles were selected through plasmid shuffling.[17] NS, not significant in a two-tailed, unpaired t test; **$p < 0.01$.

mutated alleles by both growth of wild-type and mutant strains and methionine-incorporation assays in yeast by expressing the variants in the yeast ortholog *MES1* (Figures 2A and 3A and Figures S5 and S6). Activities of the mutated alleles were compared with the wild-type and the humanized alleles in case the amino acid was not conserved between human and yeast (Table 2). Compared with those in wild-type yeast, enzyme activities in humanized alleles were not significantly different. However, compared to those of the wild-type or respective humanized alleles, both calculated division times and ^{35}S incorporation were significantly different in yeast transfected with the mutated alleles, with the exception of $mes1^{Asn325Thr}$, one of the two variants found in the individuals from Réunion. Although $mes1^{Asn325Thr}$ alone does not attenuate enzyme activity, it worsens the phenotype if it is expressed in combination with $mes1^{Ser499Leu}$. Of note, attenuation of enzyme activity occurs in a liquid medium without methionine, and its activity can be restored in a medium supplemented with 20 μg/ml methionine (Figures 2B and 3B). This observation, together with the predicted position of the mutated amino acids inside the protein, renders interference of the variants with substrate binding the most likely functional mechanism.

We have provided convincing genetic and functional evidence that *MARS* mutations are the cause of a specific type of PAP. We delineated a 530-kb candidate region by performing homozygosity mapping in 14 affected individuals from Réunion. Within this candidate region, exome and genome sequences helped to identify two rare homozygous missense variants in close proximity in a single gene, *MARS*. Three other non-synonymous variants in this region had an allele frequency of at least 0.21 and are therefore unlikely to be disease causing. The most likely disease-causing variant, p.Ser567Leu, was found in all 26 investigated affected individuals, had a frequency of only 4 in approximately 60,000 control samples in the ExAC dataset, and was not present in 4,000 in-house control samples. We further excluded structural variations in this region by genome sequencing. Next, we identified a different homozygous *MARS* missense variant in two affected siblings from Tunisia and compound-heterozygous variants, one of which was identical to the Réunion haplotype, in a French individual with sporadic PAP. The variants p.Ala393Thr and p.Ser567Leu are in strong linkage disequilibrium in the Réunion population, therefore hampering a conclusion about their causality for PAP. Functional investigation in yeast provided evidence that p.Ser567Leu is disease causing given that the corresponding mutation in yeast resulted in reduced growth and more than a 50% reduction of methionine incorporation, whereas the mutation corresponding to p.Ala393Thr did not show a phenotype. This interpretation is further supported by data from the East Asian population where the p.Ala393Thr variant is present in 19 of 8,654 alleles, whereas p.Ser567Leu is absent. However, the possibility that p.Ala393Thr contributes to the phenotype cannot be excluded, given that the corresponding mutation in yeast aggravated the phenotype of the mutation corresponding to p.Ser567Leu in a double-mutation strain. We assessed the functional impact of the mutations by conducting yeast complementation studies. Confirmation by aminoacylation assays might be worthwhile. However, the validity of yeast assays has been shown during the investigation of several other ARS mutations in which an attenuated function demonstrated in yeast was confirmed by aminoacylation assays.[18] The human genome harbors 37 ARS loci. 17 encode a cytoplasmic enzyme, 17 a mitochondrial enzyme, and 3 a bi-functional enzyme that is present in both cell compartments.[18] Thus far, ARS mutations have been implicated in autosomal-recessive mitochondrial disease and autosomal-dominant peripheral neuropathies known as Charcot-Marie-Tooth disease.[18]

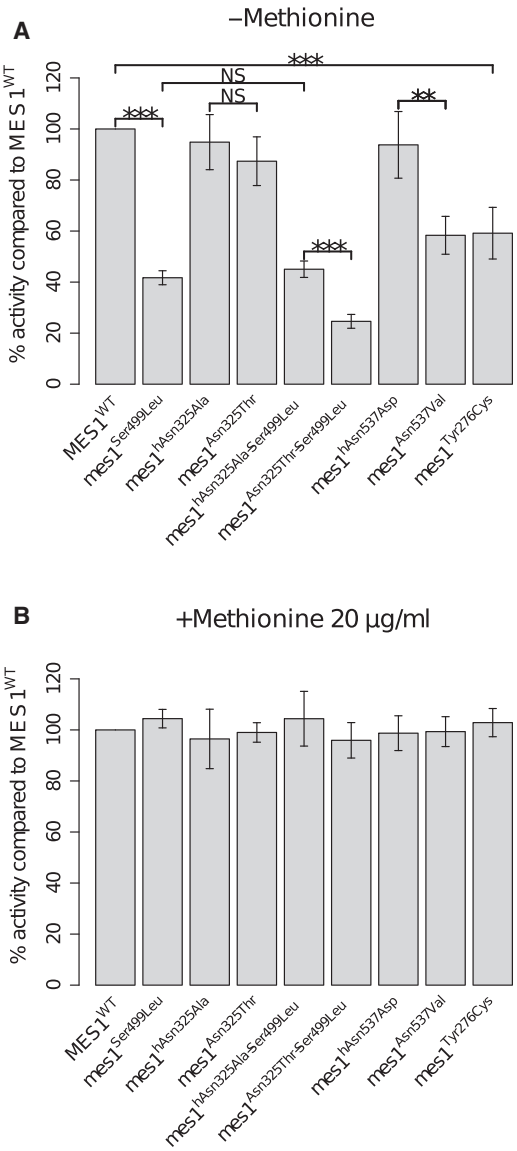

Figure 3. ^{35}S Incorporation of *MES1* Wild-Type and *mes1* Mutant Strains

Incorporation (A) without methionine or (B) with 20 µg/ml methionine. Values are normalized to the wild-type strain, which was set as 100%. Four replicates were performed for the experiments without methionine and three replicates for the experiments with methionine. The error bars indicate SDs. Cells were inoculated at a final concentration of 0.1 OD_{600}/ml in synthetic-complete-dextrose medium (0.69% yeast nitrogen base, 0.1% yeast amino acid and nucleobase mixture, 2% glucose) with or without 20 µg/ml methionine and grown at 37°C. After 16 hr, cells were diluted to a final concentration of 1.2 OD_{600}/ml. After 5 min, cells were supplemented with 1 µl, if grown without methionine, or 10 µl, if grown with methionine, of EasyTag [^{35}S]-protein labeling mix having a specific activity of 1,000 Ci/mmol (Perkin Elmer). Once we verified that the incorporation signal was linear between 2 and 10 min, we blocked protein synthesis after 6 min by adding a mix containing 200 µg cycloheximide, 1 mg erythromycin, 100 µg cold L-methionine, and 100 µg cold L-cysteine and chilling the mixture on ice. We used the trichloroacetic (TCA) method to precipitate total proteins by chilling the cells supplemented with 25% TCA on ice, then resuspended the proteins in 30 µl of 60-mM Tris-HCl (pH 6.8). For each sample, counts per minute/OD_{600} were measured on 10 µl aliquots and normalized to values for the wild-type strain, which was set as 100%. NS, not significant in a two-tailed, paired t test; **$p < 0.01$; ***$p < 0.001$.

Table 2. Variants Introduced into Mes1 for the Studies in Yeast

MARS Variant	Mes1 Variant	Humanized Mes1
p.Ala393Thr	p.Asn325Thr	p.Asn325Ala
p.Ser567Leu	p.Ser499Leu	NA
p.Asp605Val	p.Asn537Val	p.Asn537Asp
p.Tyr344Cys	p.Tyr276Cys	NA

Human transcript NM_004990.3 and yeast protein P00958 were used as reference sequences. The following abbreviation is used: NA, not applicable.

Here, we add a further phenotype to the wide spectrum of tissue-specific human diseases caused by ARS mutations. A single female infant has previously been reported to carry compound-heterozygous *MARS* mutations (c.1108T>C [p.Phe370Leu] and c.1568T>C [p.Ile523Thr]).[19] The described phenotype is compatible with that observed in this study; however, it seems to include additional signs such as acidosis, aminoaciduria, hypothyroidism, and anemia (MIM: 615486). In addition, rare heterozygous *MARS* variants have been reported in individuals affected by late-onset Charcot-Marie-Tooth disease.[20,21] In this study, however, family history of index patients in Réunion does not provide any evidence that heterozygous carriers are affected by Charcot-Marie-Tooth disease. Studies in yeast have demonstrated that attenuation of MARS activity could be rescued by supplementation with methionine. To our knowledge, a similar effect has only been described for a *VARS2* mutation before.[22] This observation suggests the need for investigation of potential beneficial effects of high-dose methionine treatment in humans.

Accession Numbers

The ClinVar accession numbers for the three sequence variants reported in this paper are SCV000196708, SCV000196709, and SCV000196710.

Supplemental Data

Supplemental Data include six figures and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.ajhg.2015.03.010.

Acknowledgments

We thank the families for participating in this study, Sandy Lösecke, Traudl Wesselack, and Andrea Schams for technical assistance, and Abdourahim Chamouine for his support in recruiting patients from Comoros Island. We thank the Centre de Ressources Biologiques de la Réunion (CRB-CHU REUNION UF1646) for providing DNA of controls. This work was supported by the German Ministry of Education and Research (01GM1113B), Else-Kroener Stiftung (MG 2013_A72), the eRARE Project 2009

(EUPAPNet), the European Register and Biobank on Childhood Interstitial Lung Diseases (European Commission, FP7, GA 305653), Telethon (GGP11011), and l'Agence Nationale de la Recherche (ANR-12-BSV1-0004-01).

Received: November 23, 2014
Accepted: March 19, 2015
Published: April 23, 2015

Web Resources

The URLs for data presented herein are as follows:

1000 Genomes, http://browser.1000genomes.org
ClinVar, https://www.ncbi.nlm.nih.gov/clinvar/
ExAC Browser, http://exac.broadinstitute.org
OMIM, http://www.omim.org/
SWISS-MODEL automated protein-structure homology-modeling server, http://swissmodel.expasy.org
UCSC Genome Bioinformatics, http://genome.ucsc.edu

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