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THIAMINE RESPONSIVE MEGALOBlastic ANEMIA: Identification of novel compound heterozygotes and mutation update

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Abstract

Objective—To determine causative mutations and clinical status of seven previously unreported kindreds with TRMA syndrome, (Thiamine Responsive Megaloblastic Anemia, OMIM #249270), a recessive disorder of thiamine transporter Slc19A2.

Study design—Genomic DNA was purified from blood, and *SLC19A2* mutations were characterized by sequencing PCR-amplified coding regions and intron-exon boundaries of all probands. Compound heterozygotes were further analyzed by sequencing parents, or cloning patient genomic DNA, to ascertain that mutations were in *trans*.

Results—We detected 9 novel *SLC19A2* mutations. Of these, five were missense, three nonsense, and one insertion. Five patients from four kindreds were compound heterozygotes, a

finding not reported previously for this disorder, which has mostly been found in consanguineous kindreds.

Conclusion—*SLC19A2* mutation sites in TRMA are heterogeneous; with no regional “hot spots”. TRMA can be caused by heterozygous compound mutations; in these cases, the disorder is found in outbred populations. To the extent that heterozygous patients were ascertained at older ages, a plausible explanation is that if one or more allele(s) is not null, partial function might be preserved. Phenotypic variability may lead to underdiagnosis or diagnostic delay, as the average time between the onset of symptoms and diagnosis was 8 years in this cohort.

Keywords

megaloblastic anemia; diabetes mellitus; sensorineural deafness; sideroblastic anemia

INTRODUCTION

Thiamine Responsive Megaloblastic Anemia (TRMA, OMIM 249270), also known as Roger’s syndrome^{1, 2}, is characterized by the clinical triad of megaloblastic anemia, non-type I diabetes mellitus and sensorineural deafness. In addition other clinical findings, such as optic atrophy, congenital heart defects, short stature, and strokes have been described in a few cases (see Table 3). The disease can manifest anytime between infancy and adolescence and often not all the cardinal findings are present initially. The bone marrow reveals an unusual combination of ringed sideroblasts and megaloblasts. Anemia typically improves significantly with pharmacological doses of thiamine (25–75 mg/day compared to US DRI of ~1.1 mg/day), although the erythrocytes remain macrocytic. Variable improvement in diabetes is also noted. However, the hearing loss is apparently irreversible, though delay in onset of deafness may be possible.

The *SLC19A2* gene, on chromosome 1q23.3, is implicated in all published TRMA patients (see Table 1). The *SLC19A2* gene encodes a 497 amino acid high-affinity thiamine transporter. This protein, named by its relation to the previously described reduced folate carrier protein - SLC19A1, is predicted to have twelve transmembrane spanning regions. The structure of the SLC19A2 protein itself has not been determined. Analysis of *SLC19A2* mutations revealed significant heterogeneity, although the majority are predicted to be null, with premature translation termination due to nonsense or frameshift mutations. All patients reported thus far have been homozygous for the mutations identified (Table 1). Here we report the first four compound heterozygous mutations and additional novel mutations identified, along with the clinical features of the patients.

MATERIALS AND METHODS

Recruitment of patients

All patients were referred to our laboratory for analysis of the *SLC19A2* gene. We obtained informed consent from either affected individuals or their parents, as appropriate and the study “Genetic Basis of Thiamine-Responsive Anemia” was approved by the Children’s Hospital Boston Committee on Clinical Investigation.

DNA extraction

Whole genomic DNA was isolated from peripheral blood of each subject using the Puregene DNA purification Kit (Gentra Systems), and quantified by spectrophotometry.

DNA Sequencing

Exons and intron-exon junctions were amplified by PCR using the genomic DNA under previously reported conditions and primers.³ The fragments were purified with Qiagen purification kits (Qiagen). We determined the sequence of both DNA strands using fluorescent dye-termination chemistry at the Children's Hospital Boston Molecular Genetics Core Facility. Sequencing results were analyzed using the program Sequencher 4.8 DNA sequence assembly software (Gene Codes). The sequence was compared with the wild type human *SLC19A2* gene (GenBank accession: AJ238413) and mutations were confirmed by independent reamplifications. The numbers for the nucleotide changes are reported in accordance with GenBank entry GeneID: 10560 with the A of the initiator methionine codon, and the lead methionine each numbered as 1.

Furthermore, the absence of the missense variants in healthy normals was confirmed in 50 unrelated individuals (i.e. 100 chromosomes) supporting the likelihood that the variants are pathogenic.

Analysis of compound heterozygotes

The phase of variants found in the compound heterozygotes was assessed by sequencing the unaffected parents when available. In one case, where the parents were unavailable for testing, the gene fragment containing both of the variants was cloned in competent E Coli using the TOPO TA cloning kit (Invitrogen). The plasmid DNA, from the different colonies was isolated using a miniprep kit (Qiagen) and sequenced to determine whether the two variants were in *cis* or *trans*.

RESULTS

Clinical presentation

In addition to the clinical triad of thiamine responsive megaloblastic anemia, diabetes mellitus and sensorineural deafness, various other features were observed, such as short stature, various forms of visual impairment, developmental and speech delay, petit mal seizures and cryptorchism. The clinical findings of the patients in our series are detailed in Table 2 and 3.

Notably, in one case (Patient 2), a compound heterozygote, the hearing is normal even at 15 years of age. Another compound heterozygote (Patient 5) has only minimal sensory-neural hearing deficit at 30 years of age. In both these cases the diagnosis of TRMA was considered even in the absence of the classical triad of symptoms as the anemia and diabetes responded well to pharmacological doses of thiamine. The diagnosis of TRMA was only considered several years after the patient first became symptomatic. This suggests that some individuals with mutations in the *SLC19A2* gene may not immediately be recognized as they do not present with the typical clinical triad and variable severity of phenotype. As already mentioned many of these patients present with additional signs and symptoms, thus the clinical presentation can be sometimes misleading.

Mutation analysis

By determining the sequence of *SLC19A2* in all probands we identified nine novel mutations amongst these seven families. Of these, five were missense mutations (Table 2). Five patients were found to be compound heterozygotes. By sequencing DNA of the unaffected parents, or cloning patient genomic DNA, the variants were confirmed to be on different alleles ("*trans*"). This is important because if they were *cis*, the patients would be predicted to have one normal allele.

DISCUSSION

We identified nine novel mutations and one previously known mutation among seven TRMA kindreds. Six of these were missense mutations, three nonsense and one insertion; these can be added to the prior 19 distinct mutations in *SLC19A2* (Table 1). These mutations are spread across the first four exons. Among 28 *SLC19A2* mutations now known in about 70 total reported patients, only four variants appear in multiple families and of these only two, 484C>T and 515G>A likely arose independently in different ethnic populations (Figure 1), while others are potentially from single founders or limited geographic regions. The majority of the mutations are null mutations as a consequence of nonsense or frameshift mutations.

Although missense mutations could conceivably yield interesting structure/function relationships, or correspond to variable phenotype, not enough data exist to make these correlations. Only five of the previously described mutations are missense, and some of those five impair proper processing and cellular localization⁴, and may be “functionally null”. In prior reports, all TRMA kindreds had homozygous mutations in *SLC19A2*, usually due to consanguinity or ethnic isolation and homozygosity by descent. However four patients in this group are compound heterozygotes (Table 2). Two of these patients present with less severe hearing phenotypes. Patient 2 has normal hearing at age 15, while Patient 5 had a first observation of mild hearing loss at age 30. It remains unclear if these milder phenotypes can be explained by compound heterozygous missense mutations that are not functionally null.

Although the clinical triad of anemia, diabetes and deafness defines the syndrome of TRMA, other clinical signs or symptoms are often observed in TRMA patients (Table 3). These associated findings also add difficulties to attempts to correlate genotype and phenotype: for example, optic atrophy, retinal changes, and stroke (or stroke-like episodes) are certainly features of TRMA, but seen only in a minority of cases, even with homozygous null mutations. Although the clinical presentation of TRMA shares some features with Wolfram Syndrome (DIDMOAD, OMIM # 222300) and a variety of mitochondrial disorders, these are known to be distinct.⁵

The pathophysiology of TRMA syndrome has been elucidated in metabolic studies in fibroblasts from patients, and in a mouse model deficient in *Slc19a2*, the murine homolog of *SLC19A2*: Megaloblastosis is due to defective *de novo* synthesis of nucleic acids, because limited intracellular thiamine affects the pentose phosphate pathway and production of ribose-5-P.⁶ Observed ringed sideroblasts and ineffective erythropoiesis in TRMA are most likely due to a decrease in succinyl-CoA, which is needed for heme biosynthesis and generated by the action of a thiamine-requiring mitochondrial enzyme, α -ketoglutarate dehydrogenase.⁷ The *Slc19a2* deficient mice become deaf due to a loss of cochlear inner hair cells.⁸ Although patients' cochleae cannot be examined as for the mice, this spatial distribution of transporters as a cause of the particular pathology is both plausible and likely. Most tissues of normal mammals express two high-affinity thiamine transporters, both *SLC19A2* and *SLC19A3*, so that if one is missing, it is supposed that the other protects those tissues (brain, heart, liver, skeletal muscle, intestine, for example). However, *SLC19A2* is the sole transporter in marrow, pancreatic beta cells, and a subset of cochlear cells^{8, 9}, and therefore deafness, diabetes and anemia are the consequences of deficiency.

A potential limitation of this survey of mutations is one of ascertainment bias. When the TRMA gene was under study in the late 1990s, investigators intentionally accumulated kindreds from consanguineous backgrounds for homozygosity mapping.² More recently, clinicians identify TRMA when confronted with the full TRMA syndrome (diabetes,

macrocytic/thiamine-responsive anemia, and hearing loss). We predict that more mildly affected patients, *either* with later onset of manifestations or without the cardinal clinical triad, remain to be discovered. We predict that to some degree, less severely affected patients may be expected to have less drastic mutations in the *SLC19A2* gene. Many adult patients are now known, but only a few presented as adults. Our study remains open to evaluate research samples from full blown TRMA, but also from subjects with only two suggestive symptoms (e.g. hearing loss and thiamine-responsive anemia, anemia with diabetes, or diabetes and deafness). It is likely that the age-range and spectrum of clinical findings in *SLC19A2*-related disorders will continue to widen as we learn more about the disorder.

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Abbreviations

TRMA	Thiamine Responsive Megaloblastic Anemia
OMIM	Online Mendelian Inheritance in Man
PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic Acid
US DRI	United States Dietary Reference Intake
DIDMOAD	Diabetes Insipidus, Diabetes Mellitus, Optic Atrophy, and Deafness
Y	Years
M	Months
BM	Bone Marrow
FS	Frameshift
TER	Termination
DEL	Deletion

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Figure 1. Known Mutations in *SLC19A2* Leading to TRMA
All 28 reported mutations are presented according to their location in the thiamine transporter gene *SLC19A2*. Squares represent the number of unique kindreds with such mutation.

Table 1

Reported mutations in *SLC19A2*

Ethnicity	Exon	Mutation	Amino Acid	Domain	Phenotype of TRMA triad*			Ref
					Anemia	Diabetes	Deafness	
African- American	1	152C>T	P51L	Extracellular	1 yr	13 m	2½ y	10
Pakistani	1	196G>T	E66X	Terminates at Extracellular	1 yr	1 yr	1 yr	4
Pakistani (Kashmir)	1	196G>T	E66X	Terminates at Extracellular	1 yr	2 y	1 yr	4
Danish	1	196G>T	E66X	Terminates at Extracellular	10 m	10 m	2 y	11
Pakistani (Kashmir)	2	242insA	Ins81fs/ter97	Fs starts Transmembrane and X in Cytoplasmic	6 y	6 m	7 m	4
Iranian	2	242insA	Ins81fs/ter97	Fs starts Transmembrane and X in Cytoplasmic	Age range: 2½ to 24 m	Age range: 2½ to 24 m	Age range: 2½ to 24 m	12
Moroccan	2	277G>C	D93H	Cytoplasmic	8 m	8 m	3 y	4
Tunisian	2	287delG	Del96fs/ter117	Fs starts Cytoplasmic and X in Transmembrane	8 m	5 y	2 y	13
Brazilian	2	428C>T	S143F	Transmembrane	1½ m	6 y	6 y	4
Iranian	2	429delTT	Del143fs/ter239	Fs starts Transmembrane and X in Cytoplasmic	Age range: 2½ to 24 m	Age range: 2½ to 24 m	Age range: 2½ to 24 m	12
Indian (Punjab)	2	473C>G	T158R	Cytoplasmic	2½ y	2½ y	6 m	14
Turkish	2	484C>T	R162X	Terminates at Cytoplasmic	3 y	3 y	3 y	4
Pakistani (Kashmir)	2	484C>T	R162X	Terminates at Cytoplasmic	2 y	2 y	Birth	15
Japanese	2	484C>T	R162X	Terminates at Cytoplasmic	1 mth	6½ y	6 y	15
Italian	2	515G>A	G172D	Transmembrane	3 y	3 y	3 y	15
Turkish	2	566_567	Insdel189fs/ter239	Fs starts Extracellular (3)	2 y	2 y	2 y	16
Turkish	2	delGC 697C>T	Q233X	Terminates at Cytoplasmic	<5 y	5 y	<5 y	17
Lebanese	2	724delC	Del242fs/ter259	Fs starts Cytoplasmic and X in Cytoplasmic	7 m	7 m	7 m	15
Israeli-Arab	2	724delC	Del242fs/ter259	Fs starts Cytoplasmic and X in Cytoplasmic	3 m	3 m	3 m	15
Indian (Punjab)	2	750G>A	W250X	Terminates at Cytoplasmic	2 y	12 y	2 y	15

Ethnicity	Exon	Mutation	Amino Acid	Domain	Phenotype of TRMA triad*			Ref
					Anemia	Diabetes	Deafness	
Alaskan	3	885delT	Del295fs/ter313	Fs starts Transmembrane and X in Extracellular	Age range: 8 m to 14 m	Age range: 8 m to 3 y	Age range: 8 m to 2 y	3**
Turkish	4	1074G>A	W358X	Terminates at Cytoplasmic	5 m	3 y	1½ y	18
Turkish	4	1105delT T	Del369fs/ter385	Fs starts Transmembrane and X in Extracellular	7 m	7 m	7 m	19
Turkish-Kurd	4	1147delG T	Del383fs/ter385	Fs starts Extracellular and X in Extracellular	NA	NA	NA	3
Lebanese	4/5	1223 + 1G>A	408+1splice	Transmembrane	2 y	6 y	1 yr	4

* Reported are the ages at onset, presentation or diagnosis of the index cases (according to reference). Other clinical features are listed in Table 3.

** Unpublished data E. J. Neufeld

m= months, y=years; fs=frameshift, ter=termination, del=deletion

Table II

Novel mutations in *SLC19A2*- Genotypes and Phenotypes

ID	Ethnicity	Exon	Mutation*	Amino Acid	Domain	Age at Dx	Anemia	Diabetes	Deafness***
1	Korean	1	121G>C	G41R	Transmembrane	4 y	11 m Hb 9.6 g/l, MCV 102 fl BM: Megaloblastosis & ringed sideroblasts	3½ y off insulin after initiation of thiamine Rx	3 y mild bilateral hearing loss, hearing aids
2	Indian	2	413G>A 758delT	E138K, Del253fs/ter260	Transmembrane Fs starts Cytoplasmic and X in Cytoplasmic	10 y	7 y** BM: Megaloblastosis & ringed sideroblasts Transfusion-dependent until thiamine Rx	10 y reduced insulin requirement after initiation of thiamine Rx	hearing normal BAER normal
3	Lebanese (Canadian)	2	454_458del GGCA Tins TA	G152X	Terminates at Cytoplasmic	13 m	1 yr BM: Megaloblastosis Hb 5.0 g/l, MCV 91 fl On Thiamine: Hb 11.0 g/l, MCV 75 fl	6½ y requires insulin	2 y sensorineural hearing deficit, hearing aids
4a	Honduran (Floridian)	2	515G>A 760insT	G172D E254X	Terminates at Transmembrane Terminates at Cytoplasmic	12 y	11y BM: Megaloblastosis On Thiamine: Hb 12.5 g/l, MCV 100 fl	5 y requires insulin	5 y Cochlear implants
4b	Honduran (Floridian)	2	515G>A 760insT	G172D E254X	Terminates at Transmembrane Terminates at Cytoplasmic	4 y	4 y BM: Megaloblastosis & ringed sideroblasts On Thiamine: Hb 12.4, MCV 88.5 fl	1½ y insulin discontinued after 1 year of thiamine Rx	5 y mild sensorineural hearing deficit
5	Italian	2/3	515G>A 1002G>A	G172D G335D	Transmembrane Extracellular	11 y	1 yr BM: mild erythroid hyperplasia Hb maintained at 10 g/l Transfusion-dependent until thiamine Rx	11 y	30 y mild sensorineural hearing deficit. BAER revealed deficit
6	Caucasian	2	602C>T 688A>T	A201V I230F	Transmembrane Cytoplasmic	31 y	7 y	6 y	8 m

ID	Ethnicity	Exon	Mutation*	Amino Acid	Domain	Age at Dx	Age at which the noted findings were documented***		
							Anemia	Diabetes	Deafness
							Hb 11 g/dl, MCV 101 fl. BM: Megaloblastosis & ringed sideroblasts. Transfusion-dependent until thiamine Rx	(after an episode of tonsillitis)	sensorineural hearing deficit
7	Portuguese	4	1172insCA T	Ins393S	Inserts Serine in Transmembrane	6 y	1 yr Hb 5.4, MCV 88fl BM: Megaloblastosis and ringed sideroblasts. Hb improvement with thiamine Rx	6 y requires insulin	6 y sensorineural hearing deficit

* Numbered from start ATG as in ref 3.

** Clinical description in Sunil et al, *Indian Pediatrics*, 2009, in press

*** In most cases age corresponds to the time when patients presented at an institution and the symptoms were noted. Therefore, it is difficult to define the actual onset of symptoms, because e.g. the onset of glucose intolerance could have preceded diabetes.

m= months, y=years

Table III

Other clinical findings in patients with TRMA

Other clinical findings in patients with TRMA	Cases (References of previous reports)
Visual impairments	
Retinal dystrophy and optic nerve atrophy	(Ref14,18–21)
-Maculopathy	Patient 3 (Ref14)
-Cone-rod dystrophy	Patient 4a (Ref22)
-Dystrophic pigmentary epithelium	Patient 5
Others	
-Astigmatism	Patient 1 (Ref14)
-Vision loss (cause unknown)	Patient 1 and 2
-Nystagmus	Patient 2 and 4a
Cardiac defects	
Atrial septal defect	Patient 3 (Ref13,23)
Ventricular septal defect	(Ref7,13,23)
Dysrhythmia/Conduction abnormalities	(Ref18,24–26)
Ebstein's anomaly	(Ref24,27)
Neurological impairments	
Seizures	Patient 1,6 (Ref26)
Developmental delay	Patient 1
Stroke	Patient 3 (Ref 24,28)
Ataxia	(Ref 29)
Others	
Short stature	Patient 1, 2, 4a (Ref 14,17,18,30)
GE reflux, vocal cord nodules,	Patient 1
Cryptorchidism	Patient 1
Situs inversus	(Ref 26)
Immune Thyroiditis	(Ref 10)