RESEARCH ARTICLE

The Spectrum of Mutations, Including Four Novel Ones, in the Thiamine-Responsive Megaloblastic Anemia Gene *SLC19A2* of Eight Families

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Thiamine responsive megaloblastic anemia (TRMA) is an autosomal recessive disorder with a triad of symptoms: megaloblastic anemia, deafness, and non-type 1 diabetes mellitus. Occasionally, cardiac abnormalities and abnormalities of the optic nerve and retina occur as well. Patients with TRMA often respond to treatment with pharmacological doses of thiamine. Recently, mutations were found in patients with TRMA in a thiamine transporter gene (*SLC19A2*). We here describe the mutations found in eight additional families. We found four novel mutations and three that were previously described. Of the novel ones, one is a nonsense mutation in exon 1 (E65X), two are missense mutations in exon 2 (S142F, D93H), and another is a mutation in the splicing donor site at the 5′ end of intron 4 (C1223+1G>A). We also summarize the state of knowledge on all mutations found to date in TRMA patients. *SLC19A2* is the first thiamine transporter gene to be described in humans. Reviewing the location and effect of the disease causing mutations can shed light on the way the protein functions and suggest ways to continue its investigation. Hum Mutat 16:37–42, 2000.

KEY WORDS: **TRMA; SLC19A2; megaloblastic anemia; diabetes; deafness; thiamine**

DATABASES:
SLC19A2 – OMIM:603941, 249270 (TRMA); GDB:9837779; HGMD:SLC19A2

INTRODUCTION

Thiamine responsive megaloblastic anemia (TRMA) is a monogenic, autosomal recessive disorder characterized by: megaloblastic anemia, diabetes which is non-type 1 in nature, and sensorineural deafness (MIM# 249270). In addition some patients also have congenital heart anomalies, arrhythmias, and/or abnormalities of the retina. Disease onset is in early childhood, and most of the patients respond to treatment with pharmacological doses of thiamine [Rogers et al., 1969; Viana

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Electronic Database Information Online Mendelian Inheritance in Man: http://www.ncbi.nlm.nih.gov/omim; ScanProsite: http:/www.expasy.ch/tools/scnpsit1.html; TMPRED: http:/www.ch.EMBnet.org/software/TMPRED_form.html

and Caravalho, 1978; Haworth et al., 1982; Mandel et al., 1984; Borgna-Pignatti et al., 1989; Grill et al., 1991; Vora and Lilleyman, 1993; Rindi et al., 1994; Bazarbachi et al., 1998]. We have recently found mutations in a novel gene, *SLC19A2* (MIM# 603941), encoding a putative transmembrane protein homologous to the reduced folate carrier proteins [Labay et al., 1999].

We report here on the spectrum of mutations found in *SLC19A2* in eight families of various ethnic backgrounds. We also summarize the information on all mutations found to date in *SLC19A2*.

MATERIALS AND METHODS

Families

We analyzed eight consangineous families, including 12 patients with TRMA and their 24 healthy parents and siblings (see Fig. 1). The families come from diverse ethnic origins (see Table 1).

An informed consent was collected from all subjects or their legal guardians in accordance with the local authorities.

Controls

We tested between 51 and 100 controls from 35 unrelated families of Israeli-Arabs and Ashkenazi Jews as well as two Ceph families, for the presence of all mutations found. We included positive controls (the affected patient and a heterozygote parent) in every batch of controls that was tested to verify the assay was properly performed. The testing was done by sequencing, restriction enzymes, and/or allele specific PCR (ASPCR).

Sequencing

We sequenced the six exons of *SLC19A2* in all subjects. Once a mutation was identified, we sequenced the mutated exon again in all subjects, their parents, and some siblings to verify the results. We also sequenced the mutated exon in the controls where other methods of verification were not available. We amplified all exons by PCR using primers we designed [Labay et al., 1999]. The total reaction volume was 30μl containing 200 ng genomic DNA, 1μM of each primer, 200 μM of each dNTP, 1 U of Taq polymerase, and 3 μl amplification buffer (10X) provided by the New England Biolabs® (Beverly, MA, USA) PCR conditions were: denaturation at 94°C for 4 min; 35 cycles of 94°C, anealing temperature, and 72°C for 30 sec each; and a final step at 72°C for 7 min. We analyzed the PCR products on 2% agarose gel and purified them using the Qiaquick™ gel extraction kit by Qiagen® (Chatsworth, CA, USA). We se-

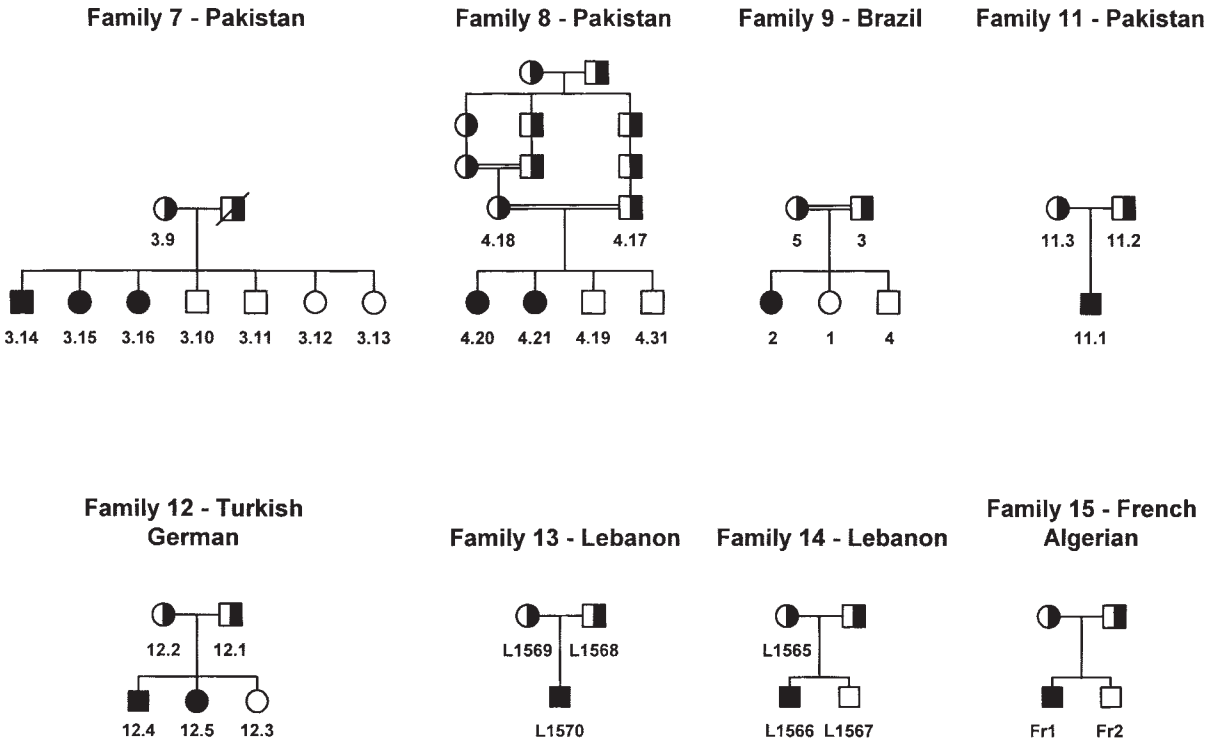

FIGURE 1. **Pedigrees of tested individuals in the eight families. Numbers under the subjects are ID numbers.**

TABLE 1. Mutations Found to Date in *SLC19A2*

Family	Ethnic origin	Mutation	Amino acid	Family clinical description
7	Pakistan	196G>T	E65X	Haworth et al., 1982
8	Pakistan	196G>T	E65X	Vora and Lilleyman, 1993
11*	Pakistan	242insA	ins81fs/ter97	Freisinger et al., 1999
3 families*	Iran	242insA	ins81fs/ter97	Vossough et al., 1995
15	France	277G>C	D93H	Grill et al., 1991
9	Brazil	428C>T	S142F	Viana and Caravalho, 1978
1 family*	Iran	429delTT	Del143fs/ter239	Vossough et al., 1995
12**	Turkey	484C>T	R162X	Freisinger et al., 1999
1 family	Pakistan	484C>T	R162X	Barrett et al., 1997
1 family	Japan	484C>T	R162X	Morimoto et al., 1993
1 family**	Italy	515G>A	G172D	Borgna-Pignatti et al., 1989
14**	Lebanon	724delC	Del24fs/ter259	Bazarbachi et al., 1998
2 families**	Israeli-Arab	724delC	Del242fs/ter259	Mandel et al., 1984; Rindi et al., 1994
1 family**	India	750G>A	W250X	Raz et al., 1998
1 family***	Alaska	885delT	Del295fs/ter313	Neufeld et al., 1997
1 family***	Turkish- Kurdish	1147delGT	Del383fs/ter385	Neufeld et al., 1997
13	Lebanon	1223+1G>A	408+1splice	Bazarbachi et al., 1998

Previously described mutations: *, Diaz et al., 1999; **, Labay et al., 1999; ***, Fleming et al., 1999.

quenced the PCR product using the Big Dye® terminator cycle sequencing kit (Perkin Elmer, Norwalk, CT, USA), and capillary electrophoresis on an ABI PRISAM 310® automated sequencer (Perkin Elmer).

Allele Specific PCR

We designed two ASPCR assays, one for the Family 14 mutation (Del242fs/ter259) and one for the Family 15 mutation (D93H). The PCR conditions are described above (see Sequencing). We used four primers for each reaction. For Del242fs/ter259 we used TRMA2B_F 5′-ACAGCCACTGAAATTGCCTA-3′, TRMA2B_R 5′-AGATCTACCAAGAGGGAGTTT-3′, TRMA2B_M 5′-GCATTGTTACTGACACCCA-3′, and TRMA2B_WT 5′-GGTGGTTAGAAGCTGGGGT-3′, and annealing temperature of 58°C. For D93H we used: TRMA2AF 5′-AGATCTTTGAGGTATTTGTAG-3′, TRMA2AR 5′-ACACAGGTAAGAGAGATGACA-3′, FR_AS_WT 5′CC TGTGTTCCTTGCCACAG-3′, and FR_AS_M 5′AGGTTTATAACGGAGGTAGTG-3′ and annealing temperature of 60°C (see result section for more detail). We used the subject and his or her parents as positive controls with every ASPCR.

Restriction Enzymes

Four mutations: 428C/T, 484C/T, Ins81fs/ter97, and 1223+1G/A caused either the creation or elimination of an endonuclease restriction site. Controls, patients, and their parents were tested for the presence of these mutations using the endonucleases BfaI, TaqI, MseI, and NdeI, respectively. We used 10μl PCR product (see Sequencing for PCR conditions), 3μl of the 10X enzyme buffer, and 1μl enzyme (5, 20, 4, and 20 units for BfaI, TaqI, MseI, and NdeI, respectively). We also added 3 μg BSA for the Taq1 and MseI reactions. We incubated the reactions overnight at 37°C except for Taq1, which was incubated at 65°C. The PCR product we used was purified for the BfaI reaction using the Qiaquick gel extraction kit by Qiagen. We used the patient with the mutation and his or her heterozygous parent as positive controls for every group of controls tested with restriction enzymes.

Haplotype Construction

We constructed haplotypes for all patients studied and their families. All haplotypes consisted of at least three informative markers. We genotyped five markers for the patients in families 7 and 8 (TMG1059, D1S433, TMG968, TMG206, and TMG86). We previously genotyped additional markers as well, and constructed extensive haplotypes for the members of family 9 (see result section). We used three markers for family 11 (TMG206, TMG86, and TMG738C), four markers for families 12 and 14 (D1S1569, TMG206, TMG86, and TMG780), four markers for family 13 (D1S1569, TMG206, TMG780, and TMG738B), and three markers for family 15 (D1S1569, TMG206, and TMG86). The TMG markers we used were previously developed by us [Labay et al., 1999], and the D1S markers were described by Dib et al. [1996]. For genotyping, we used a non-radioactive labeling procedure with the Amersham ECL system [Gyapay et al., 1996].

Computer Analysis

We used the TMPRED program to predict the orientation and location of transmembrane re-

gions, and the ScanProsite program to compare protein sequences and to identify sequence patterns.

RESULTS

We sequenced the full 6 exons of the *SLC19A2* gene, including the splicing regions, and found only one mutation per patient. We found seven mutations in eight families checked (see Table 1). Four of these mutations are novel: one is a nonsense mutation in exon 1 (E65X), two are missense mutations in exon 2 (D93H, S142F), and one is in the splicing site at the 5′ end of intron 4 (G408+1A). We also found three mutations that were previously described. Two are frame shift mutations: Ins81fs/ter97 (previously reported as 242-243insA), Del242fs/ter259 [Diaz et al., 1999] and [Labay et al., 1999] respectively, and one is a nonsense mutation: R162X [Labay et al., 1999]. The existence of all mutations was verified by repeat sequencing of patients and their parents. In addition, for six of the seven mutations an alternative method was used as well, either allele specific PCR or restriction enzyme digestion as described above.

Polymorphic markers in the vicinity of the *SLC19A2* gene segregated with the disease in the informative families (not shown). All affected individuals were homozygous for the markers tested, as expected for consanguineous families. The patient in family 9 was found previously to be homozygous along a 16 cM region surrounding the *SLC19A2* gene [Raz et al., 1998]. We also compared the haplotypes of patients of different families who were found to have identical mutations to test whether they share a common ancestor (see Fig. 2). Families 7 and 8 share the mutation E65X, and were previously found to have identical alleles at D1S2658 and D1S1569 [Raz et al., 1998]. We genotyped an additional five markers in the vicinity of these two markers (TMG1059, D1S433, TMG968, TMG206, and TMG86) and found that the two families had identical haplotypes (Fig. 2 shows the markers close to *SLC19A2*). We constructed haplotypes for the patients in Families 12 (R162X), and 14 (Del242fs/ter259) and compared them to the haplotypes of the families having identical mutations described previously [Labay et al., 1999]. Haplotypes of patients of Family 12 (Turkish origin) were different from the haplotypes of the families of Japanese and Pakistani origins, previously described. However, the haplotype of Family 14, a Lebanese family, was identical to the haplotype described previously in two Israeli-Arab families [Labay et al., 1999]. We had no DNA from the Iranian family, previously described by Diaz et al. [1999]. We therefore could not investigate whether they share a common ancestor with Family 11 having the same mutation.

We designed ASPCR tests for two of the mutations, Del242fs/ter259 and D93H, using four primers in each reaction. In the Del242fs/ter259 reaction, the primer TRMA2B_M only binds to mutant DNA because of its 3′ sequence ACCCA, which differs from the wild type ACCCCA sequence. This primer coupled with the TRMA2B_R primer produces a 188 bp product. The primer TRMA2B_WT binds only to the wild type DNA. Coupled with the TRMA2B_F primer, this primer produces a 337 bp product. In addition, a 493 bp product is produced by the primers TRMA2B_F and TRMA2B_R. We tested control patients and their parents using this system. The patients and their heterozygote parents showed the expected bands on 2% agarose gel. Wild type controls appeared as a single strong 337 bp band. (The 493 bp band does not appear in the wild type probably because TRMA2B_WT competes with TRMA2B_R.) In the D93H reaction the primer FR_AS_M only binds to mutant DNA because the last nucleotide of the primer (3′), a guanine, is specific to the mutation, while the FR_AS_WT primer only binds to wild type DNA. FR_AS_M coupled with TRMA2AF produces a 202 bp product, and FR_AS_WT coupled with TRMA2AR produces a 333 bp product. The two primers TRMA2AF and TRMA2AR produce an additional, faint, 496 bp product. Controls, patients, and their parents showed the expected bands.

All control subjects tested using sequencing, enzyme digestion, and ASPCR were found free of all mutations.

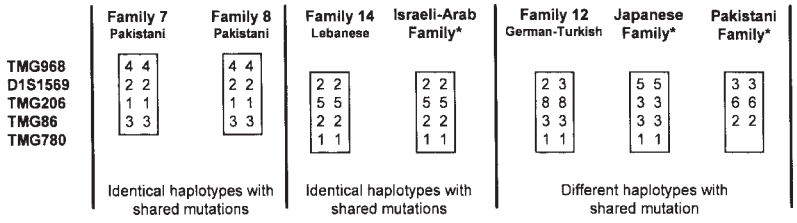

FIGURE 2. **Haplotypes covering the area surrounding *SLC19A2* of patients constructed to test whether families share a common ancestor. TMG206 is intragenic. The three groups of families tested are separated by vertical lines (see text). Extended haplotypes shown in Labay et al. [1999].**

DISCUSSION

We searched for mutations in the *SLC19A2* gene in eight families of patients affected with TRMA, of various ethnic origins. We found seven mutations all together, four of which were not previously described (see Table 1). Of the novel mutations, one is a nonsense mutation, two are missense, and one is a mutation in a splice site. Patients 14, 15, and 16 of family 7, and patients 20 and 21 of family 8 all have the same nonsense mutation (E65X), and these two Pakistani families share the same haplotype. The haplotypes of the area close to the gene are shown in Figure 2.

We found two novel missense mutations. One is in patient Fr1 of family 15 (French-Algerian) causing an amino acid change from aspartic acid to histidine. This region was predicted to be a transmembrane region. This change from an acidic to a basic amino acid may interfere with the passage of the protein through the membrane (see Fig. 3). We found a missense mutation (S142F) in Family 9 (Brazil) causing a change from serine, a hydrophilic amino acid, to phenylalanine, a hydrophobic one. Both of these missense mutations are in well conserved positions in human, mouse, and hamster RFC like proteins [Labay et al., 1999]. The fact that these missense mutations are disease causing implies that this region is critical for the protein function. We also found a mutation in the donor splicing site at the 5′ end of intron 4 (408+1splice) in the patient from Family 13 (Lebanon). Although we could not test its effect on the transcript, this mutation is likely to cause a dramatic change in the protein. This mutation is just upsream from a G protein receptor signature sequence (ScanProsite) and may thus impair the ability of the *SLC19A2* protein (Thtr1) to interact with a G protein.

We found three mutations that were described previously. Patient L1566 of Family 14 (Lebanon) has a mutation (Del242fs/ter259) that was previously found in two Israeli-Arab families [Labay et al., 1999], and the affected patient shares a haplotype around SLC19A2 with the two Israeli families (see Fig. 2). Therefore, the three families, which are all Arab families residing in geographical proximity to each other (two Israeli families and one Lebanese) probably share a common ancestor. Patients of family 12 (a Turkish family) also have a mutation that was previously described in families from Japan and Pakistan (R162X) [Labay et al., 1999], but these three families do not share a common haplotype (see Fig. 2), and are very unlikely to share a common ancestor. This mutation affects a CG pair that is likely to be a mutation hot-spot. The patient of family 11 also has a mutation (ins81fs/ter97) that was previously described by Diaz et al. [1999]. It is possible that these two families have a common ancestor since family 11 is Pakistani and the family described by Diaz et al. [1999] is from Iran. We found no correlation between the severity of disease symptoms and the type of mutation found.

Figure 3 illustrates the position of all the 12 mutations in *SLC19A2* described to date in 20 families with TRMA. We describe four novel mutations here. We have previously found four mutations in a Pakistani, Japanese, Indian, Israeli, and an Italian family [Labay et al., 1999)],. Another two were described by Fleming et al. [1999] in an Alaskan and a Turkish family, and another two mutations were found by Diaz et al. [1999] in three Iranian families. All mutations found in the eight families described here are summarized in Table 1.

All together three missense mutations were found (D93H, S142F, and G172D), all located in exon two (see Fig. 3), suggesting that this is a critical region of the protein. Two of these mutations

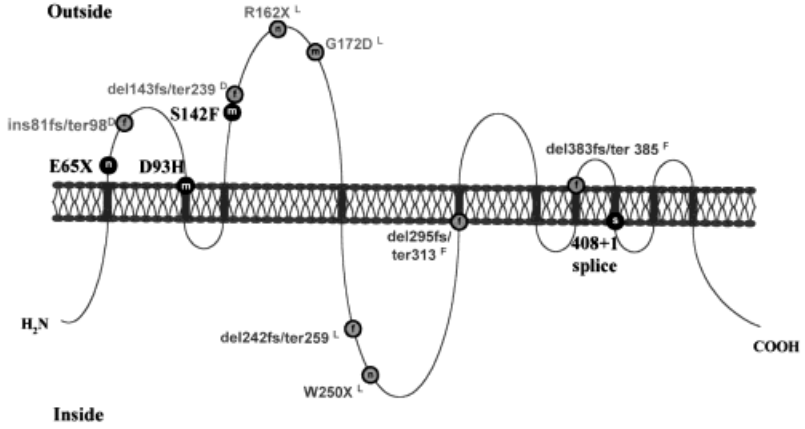

FIGURE 3. **The position of all mutations found to date in the predicted topology of *SLC19A2* (TMPRED program). In black are the four novel mutations described here. In gray are mutations previously described: D-Diaz et al. [1999] (reported as 242-243insA and 429-430delTT); L-Labay et al. [1999]; F- Fleming et al. [1999], (reported as 885delT and 1147-delGT). n is for nonsense mutation, m for missense mutation, f for frame shift, and s for a mutation in a splice site.**

(S142F and G172D) occur in an extracellular area of the protein, which thus may be responsible for thiamine recognition.

Exon 5 includes a G protein receptor consensus sequence, which suggests that this intracellular region may interact with other proteins inside the cell. So far, no mutations were found downstream of exon 4.

The types and locations of disease causing mutations in SLC19A2 can shed light on the gene's function. The existence of missense mutations in exon 2 suggest this exon has an important role, perhaps in the binding of thiamine, and can help in the design of functional studies of the Thtr1 protein. The identification of mutations in the affected families also gives the immediate benefit of providing them with an efficient and accurate means to perform prenatal diagnosis. TRMA is a rare disease of considerable interest. An increasing number of disease causing mutations has now been identified pointing out the regions of functional significance of the encoded protein. This open the ways to further functional studies.

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