Mutations in *POMT1* Are Found in a Minority of Patients With Walker–Warburg Syndrome

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Walker–Warburg syndrome (WWS) is an autosomal recessive disorder of infancy characterized by hydrocephalus, agyria, retinal dysplasia, congenital muscular dystrophy, and over migration of neurons through a disrupted pial surface resulting in leptomeningeal heterotopia. Although previous work identified mutations in the *o*-mannosyl transferase, *POMT1*, in 6 out of 30 WWS families [Beltran-Valero de Bernabe et al., 2002], the incidence of *POMT1* mutations in WWS is not known. We sequenced the entire coding region of *POMT1* in 30 consecutive, unselected patients with classic WWS. Two novel heterozygous mutations were found in two patients from non-consanguineous parents, whereas 28 other patients failed to show any *POMT1* mutations. One patient was found to be heterozygous for a transition, g.1233T > A, which predicts p.Y352X. A second patient was found also to be heterozygous for a transition g.1790C > G, which predicts p.S537R. As an additional determination of the frequency of the *POMT1* mutations in WWS, we tested for linkage of WWS to *POMT1* in six consanguineous families. All six demonstrated heterozygosity and negative LOD scores at the *POMT1* locus. From these data we show that *POMT1* is an uncommon cause of WWS, the incidence of coding region mutations in this population of WWS being less than 7%. We conclude that while the incidence of *POMT1* mutations in WWS can be as high as 20% as reported by Beltran-Valero de Bernabe et al. [2002] and it can be as low as ~7%, as reported here. © 2005 Wiley-Liss, Inc.

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INTRODUCTION

Walker–Warburg syndrome (WWS, MIM 236670) is a rare autosomal recessive disorder with developmental malformations of the brain, skeletal muscle, eyes, as well as other abnormalities [Walker, 1942; Warburg, 1978; Dobyns et al., 1985]. Affected infants present with hypotonia, blindness, or with severely diminished vision, severe developmental delay, and death during infancy [Dobyns et al., 1989b]. They suffer from congenital muscular dystrophy and congenital ocular abnormalities, which can include microophthalmia, optic nerve hypoplasia, retinal dysplasia, and cataracts [Kuchelmeister et al., 1993; Laverda et al., 1993]. Diagnosis is confirmed by MRI analysis, which reveals severe hydrocephalus, complete loss of cerebral gyration (folding), and cerebellar and brainstem hypoplasia [Barkovich et al., 1991; Cormand et al., 2001]. Approximately one-third of patients have an occipital encephalocele [Dobyns et al., 1989a]. Postmortem examination of the brain in eight cases reveals a cobblestone surface resulting from a large number of undifferentiated neurons migrating through the pial surface, forming leptomeningeal heterotopia in the subarachnoid space [Kimura et al., 1993; Squier, 1993; Vaisar et al., 2000; Beltran-Valero de Bernabe et al., 2002].

WWS appears to be a genetically heterogeneous disorder with a world-wide distribution [Beltran-Valero de Bernabe et al., 2002]. In a previous study 6 out of 30 unrelated WWS patients were shown to have loss of function mutations in the *o*-mannosyltransferase, *POMT1* [Beltran-Valero de Bernabe et al., 2002]. These results suggest that the frequency of *POMT1* mutations in WWS may be as high as 20%. We undertook this study to examine the incidence of *POMT1* mutations in a consecutive unselected series of patients with classic WWS.

MATERIALS AND METHODS

Blood and/or DNA was collected from patients for whom the diagnosis of WWS was confirmed by the following criteria: death in early childhood, ocular abnormalities and signs of congenital muscular dystrophy, severe hydrocephalus and type II lissencephaly with global agyria [Dobyns, 1989; Cormand et al., 2001]. Twenty-seven of the thirty patients in this study were reported to have all of these findings as well as one or more of the following: cerebellar hypoplasia (25), brainstem hypoplasia (21), agenesis of the corpus callosum

(22), agenesis of the septum pellucidum (14), interhemispheric fusion (11), and the presence of an encephalocele (7). One family (family 1) has been described in a previous study [Rodgers et al., 1994].

For three of the thirty patients in this study, clinical data regarding muscular dystrophy was unavailable. For one of these three clinical data for ocular abnormalities had also not been obtained. All three were reported to demonstrate severe hydrocephalus, type II lissencephaly with global agyria, and a hypoplastic cerebellum. Two of these three were reported to have agenesis of the corpus callosum and the third, a hypoplastic brainstem. Together, these CNS abnormalities are specific to WWS and thus confirm the diagnosis of WWS in these three patients.

WWS patients in this study included people of Asian, African, and Caucasian descent. Consanguineous families studied were from South Carolina, USA (family 1), Israel (family 2), Saudi Arabia (family 3), Mexico (family 4 and 5), and the United Arab Emirates (family 6) (Fig. 1). Eight of the thirty WWS patients used in this study may have been analyzed for mutations in *POMT1* in part or fully by Beltran-Valero de Bernabe et al. [2002]. However, we have re-sequenced all of these in this study because (1) we are uncertain whether *POMT1* gene sequencing had been completed in these samples and (2) including them provided a consecutive series of un-selected WWS patients that allows for more accurate data about incidence.

Informed consent was obtained from all patients and/or their parents, in accordance with protocols approved by the institu-tional review boards of Beth Israel Deaconess Medical Center, Boston Children's Hospital, local institutions, and the Office of Human Research Protection. Genomic DNA was extracted from peripheral blood lymphocytes using standard techniques. For the affected patients from family 1, DNA was extracted from paraffin-embedded brain tissue using the corresponding Qiagen QIAamp DNA mini kit and protocol.

Amplification and analysis of fluorescently labeled micro-satellite markers were performed using standard methods. The following markers D9S290, D9S752, D9S1861, D9S1863, D9S179, D9S1830, and D9S164, all within less than 4 cM from the *POMT1*, were analyzed for homozygosity. Primer sequences and conditions were obtained from the UCSC genome database (July 03 version), and are available upon request. Multipoint LOD scores were calculated using GeneHunter [Daly et al., 1998]. We assumed a susceptibility allele with frequency 0.001 and a recessive mode of inheritance with penetrance 0.999. The most current Marshfield map was used to determine distances between markers. For each marker, four equal alleles were assumed. However, the use of different allele frequencies and susceptibility allele frequencies did not significantly alter the final results.

Primers for all 19 coding exons of *POMT1* were designed to amplify exon sequences as well as 50 base pairs of adjacent intron sequences. Primers and sequences are available on request. Over 100 base pairs of both the 3′ and the 5′ UTR were also sequenced and analyzed. Amplicons generated were purified using Princeton Separation columns. Amplicons were then sequenced in both directions using an ABI3700 capillary
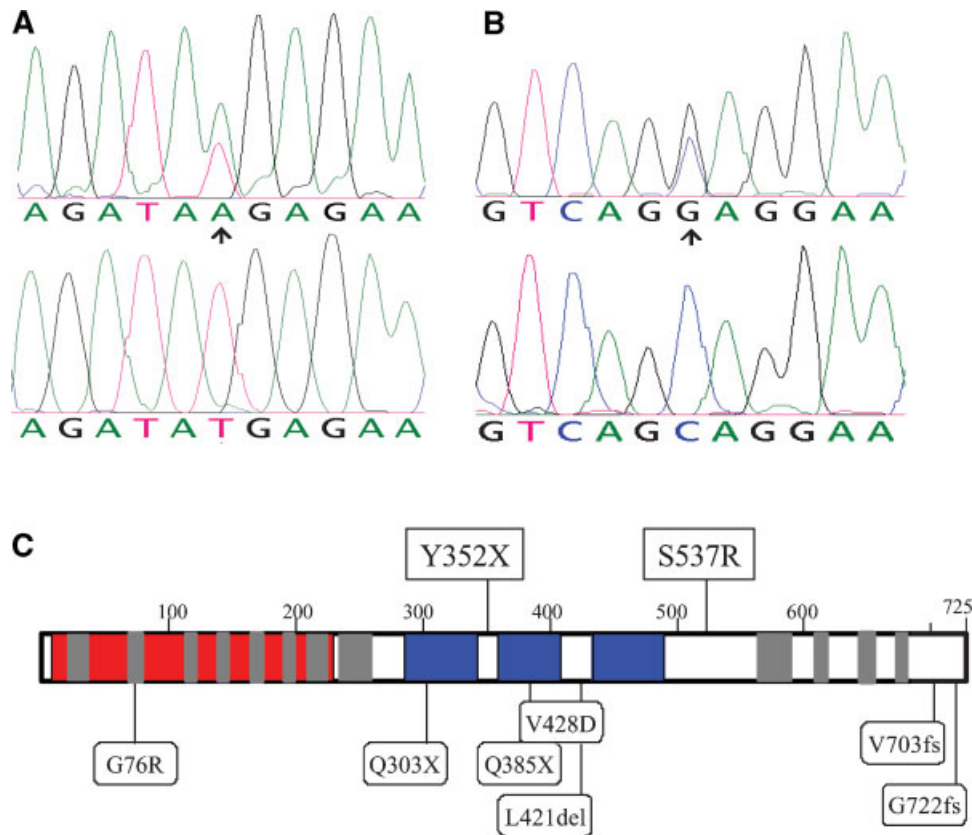

Fig. 1.   Two novel mutations disrupt conserved domains of *POMT1*. In Patient 7 a chromatogram of 12 nucleotides in exon 11 reveals a heterozygous g.1233T > A, which predicts p.Y352X (**A**, **upper panel**). Her mother does not carry this mutation (A, **lower panel**). Chromatograms of exon 16 indicate a heterozygous g.1790C > G, which predicts p.S537R, in Patient 8 (**B**, **upper panel**) but not the control sample (B, **lower panel**). Mutations are indicated with an arrow. A schematic of *POMT1* gene structure and mutations to date (**C**). MIR domains (blue) are thought to be involved in ligand transfer function. PMT domain (red) represents the family of dolichyl-phosphate-mannose-protein mannosyltransferase proteins responsible for *o*-linked glycosylation of proteins. Hypothesized transmembrane spanning regions are indicated in gray. Mutations indicated below the protein were identified in previous studies.

sequencer (Perkin Elmer Applied Biosystems, Foster City, CA). Sequence data were analyzed independently by two separate individuals.

RESULTS

Thirty non-selected, non-related WWS patients with clinical findings typical of WWS were used for this study. In each of these patients, the coding region of *POMT1* was then sequenced and analyzed both in the forward and reverse directions. Twenty-eight of the 30 patients (93.3%) failed to reveal any mutations in *POMT1*. Two novel mutations were identified in two patients from non-consanguineous families. The first patient (whom we will refer to as patient 7) is a Caucasian girl from Iowa. She was reported to have congenital muscular dystrophy, extremely elevated creatin kinase (39,650 U/L), optic atrophy, retinal dysplasia, and non-attachment. MRI analysis revealed complete agyria, kinking of the cervical medullary junction, and Dandy–Walker malformation with severe obstructive hydrocephalus involving the lateral and third ventricles. She suffered from seizures, poor coordination, had a poor sucking reflex, and difficulties swallowing, resulting in an inability to feed. She also had congenital contractures of her right wrists.

Patient 7 was found to be heterozygous for a genomic transition, g.1233T > A, which is predicted to result in p.Y352X (Fig. 1A). A stop codon at tyrosine 352 in exon 11 is then predicted to result in truncation of more than half of the *POMT1* protein and the loss of several conserved mannosyl-transferase IP2R and RyR domains (MIR) thought to be involved in ligand transfer (Fig. 1C). Sequencing of the coding regions of *POMT1* revealed only one mutation in this patient. We assume that Patient 7 is a compound heterozygote for a second mutation not in the coding region of *POMT1*.

The second patient (whom we will refer to as Patient 8) is a Caucasian boy also from the United States. He was a dysmorphic neonate with microcephaly, a sloping forehead, small eyes, micrognathia with intact lips, low set ears, and undescended testicles. Patient 8 suffered from severe seizures, poor growth, and no spontaneous respiration, requiring a ventilator. Ocular abnormalities included retinal dysplasia and anterior chamber defects. He was reported to have agyria with ventriculomegaly and Dandy–Walker malformation.

Patient 8 was found to be heterozygous for a genomic transition, g.1790C > G, which is predicted to cause the replacement of a conserved serine 537 by an arginine in exon 16 (Fig. 1B). Amino acid alignment showed that Ser537 is conserved in the homologous protein *POMT2*, as well as in *POMT1* orthologues in *Mus musculus* and *Rattus norvegicus*. It is not conserved in more distantly related orthologues in *Drosophila melanogaster* or *Saccharomyces cervevisiae* *POMT1* [Jurado et al., 1999]. Therefore, we interpret this as a likely mutation though conclusive demonstration of this would require biochemical analysis. As with Patient 7, only one mutation was identified in this patient despite extensive re-sequencing.

Neither of these changes has been previously identified in WWS patients. Neither has been reported before as a SNP, and sequencing of 110 normal control subjects of Caucasian (93) and Arabic (17) origin failed to reveal these same changes, suggesting that they are not common polymorphisms.

In addition to these two likely mutations, a total of six sequence alterations were identified in seven different patients, as listed in Table I. Two of these are silent changes that do not alter amino acids, and the other four are known single nucleotide polymorphisms and are thus also assumed to be non-pathogenic.

Six of the WWS patients showed consanguineous parents (Fig. 2) and therefore were informative enough to obtain linkage information. Analysis of seven consecutive microsatellite markers within 4 cM of the *POMT1* loci at 9q34.1 ruled out linkage to the *POMT1* locus in all six consanguineous families (Figs. 2 and 3). None of the consanguineous families showed identity by descent at the *POMT1* locus (Fig. 2) and all the LOD scores were negative (Fig. 3). This suggests that in these six families, neither coding nor non-coding mutations in *POMT1* are responsible for WWS.

DISCUSSION

Here we sequenced *POMT1* in 30 consecutive WWS patients and found two that are heterozygous for g.1790C > G (predicted p.S537R) and g.1233T > A (predicted p.Y352X) changes. These changes are likely to be mutations for the following reasons. First, sequencing of 110 control subjects failed to identify either of these same changes. Second, p.Y352X is predicted to result in severe truncation of more than half of the *POMT1* protein and p.S537R is predicted to result in the replacement of a conserved serine with a positively charged arginine. Although it is outside of any recognized domains, serine 537 is in a large central hydrophilic stretch [Jurado et al., 1999] (Fig. 1C). Around this region *POMT1* is otherwise composed of 7–12 transmembrane domains, which make up more than half of the protein length.

Our inability to find a second mutation in these two patients is not uncommon for other recessive disorders [Taniguchi et al., 2003], but points to the possibility of mutations in the non-coding regions of *POMT1*. Mutations occurring in the promoter or non-coding regions cannot be ruled out in any of the patients from non-consanguineous patients. However, although non-coding mutations might explain a small number of cases, the fact that our patients from consanguineous families failed to show linkage to *POMT1* makes it unlikely that non-coding mutations in *POMT1* are a major cause of WWS. In addition, most patients with the closely related disorder, muscle eye brain (MEB) disease, have been found to have mutations within coding exons of the causal gene *POMGnT1* [Yoshida et al., 2001; Taniguchi et al., 2003].

TABLE I. Single Nucleotide Polymorphisms of *POMT1*

Patient number	Location	Nucleotide	Amino acid	Allele frequency	Reference[b]
19, 28	Exon 8	930C > T	R251W	16.7%	rs3887873
8[a], 33[a], 41[a]	Exon 8	931G > A	R251Q	16.7%	rs2296949
8[a], 33[a], 41[a]	Exon 10	1121C > C/T	Silent T314T	16.7%	—
33, 41	Exon 10	1158G > G/A	V327I	10%	rs4740164
8[a], 33[a], 41	Exon 11	1292C > C/T	Silent D371D	16.7%	rs3739494
23, 30	Exon 17	1937G > A	Silent R586R	10%	—

Nucleotide number is based on the POMT1 reference sequence (GeneBank Accession Number NM_00717).
[a]Nucleotide alterations found in the heterozygous state. All others were homozygous.
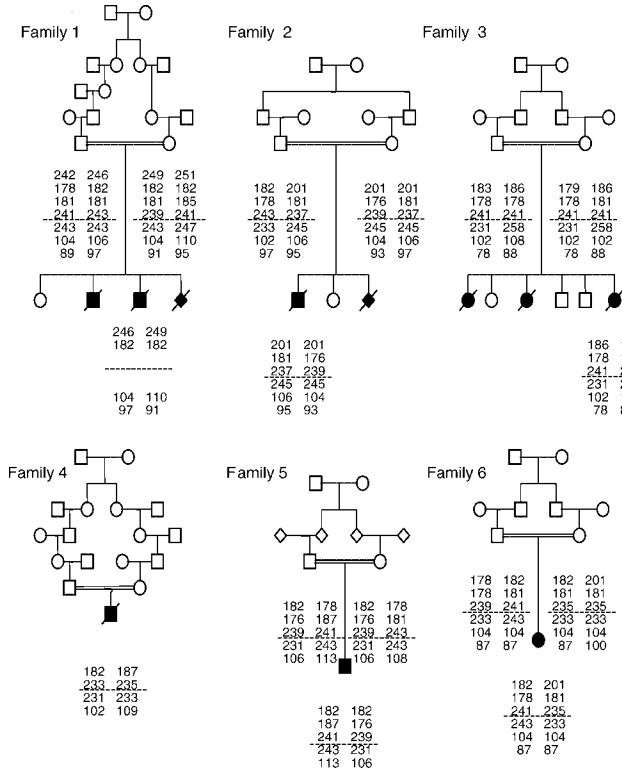[b]Referenced in NCBI SNP Cluster Report.

Fig. 2. WWS patients from six consanguineous families failed to show homozygosity at the *POMT1* locus. Six microsatellite markers less than 4 cM from the *POMT1* loci rule out identity by descent. Alleles for the following markers are listed in the following order based on physical distance: cen-D9S290, D9S752, D9S1861, D9S1863, D9S179, D9S1830, D9S164-tel. Allele numbers represent relative but not absolute sizes as determined by ABI 3100 capillary analysis, Genescan and Genotyper programs version 3.7. Horizontal dashed bars between alleles for D9S1863 and D9S179 indicate the location of *POMT1*. Gray alleles indicate homozygosity. For the affected from family 1, several markers failed to amplify due to the fact that DNA was extracted from paraffin embedded tissue.
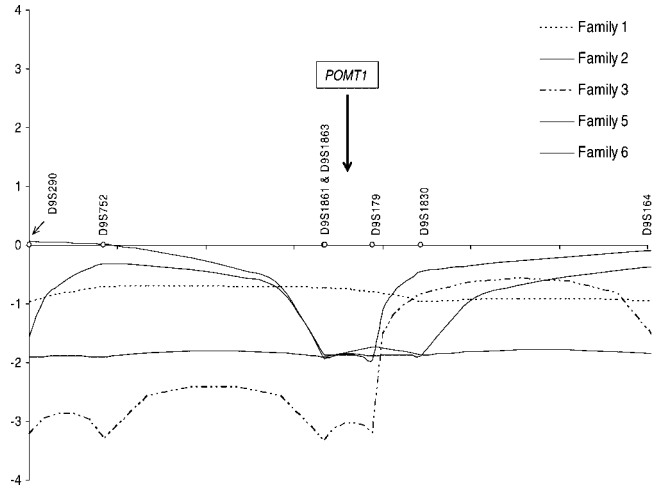

Fig. 3.   Multipoint LOD scores rule out linkage at *POMT1*. LOD scores were calculated for families 1, 2, 3, 5, and 6 using markers cen-D9S290, D9S752, D9S1861, D9S1863, D9S179, D9S1830, D9S164-tel at 9q34. D9S290 was arbitrarily set as the zero point on the map. The relative map positions are shown along the X-axis with tick marks indicating 1 cM distance increasing from left to right, from 0 to 7 cM. The multipoint LOD scores are shown on the Y-axis. The locations of the markers used in the multipoint analysis are indicated.

Our results suggest that mutations in *POMT1* represent a small subset of WWS patients (just under 7% assuming the patients with heterozygous mutations carry second mutations as well). In a previous study, five novel mutations in *POMT1* were identified in six unrelated families [Beltran-Valero de Bernabe et al., 2002]. The families with *POMT1* mutations were from various regions of Europe including Italy, Holland, Austria, and Turkey. Thus, the mutations found were not from a specific geographic region nor did they appear to represent a specific founder mutation. Our own WWS patients derive from a broad range of geographical and ethnic origins and include many of European ancestry. Hence, geographic selection is an unlikely explanation for the apparent low frequency of *POMT1* mutations in our patients.

The difference in *POMT1* mutation frequency in this study and the previous study [Beltran-Valero de Bernabe et al., 2002] could be due to clinical differences in the WWS populations selected for sequencing. However, the criteria we used for diagnosis of WWS in our study closely resembles those described in the previous publication. In addition, a second study has identified a deletion in *POMT1* in a Japanese boy with a slightly atypical presentation of WWS suggesting that *POMT1* mutated WWS patients can have a broad range of phenotypes [Kim et al., 2004]. A detailed clinical and radiologic comparison between WWS patients with and without *POMT1* mutations are needed in order to determine that how these two groups of WWS patients might differ.

In conclusion, our data confirm the presence of *POMT1* mutations in WWS patients, but suggest that, in our patient population, *POMT1* mutations cause a small fraction of the cases and that the predominant gene or genes that cause WWS remain unidentified. We conclude that while the incidence of *POMT1* mutations in WWS can be as high as 20% as reported by Beltran-Valero de Bernabe et al. [2002] and it can also be as low as ~7%, as reported here. Although DNA based sequencing may be useful in limited settings, families and physicians seeking testing for *POMT1* mutations should be cautioned of the potential low return of DNA based sequencing for diagnostic purposes.

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