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# Mutations in the Lysosomal Enzyme–Targeting Pathway and Persistent Stuttering

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## Abstract

**BACKGROUND—**Stuttering is a disorder of unknown cause characterized by repetitions, prolongations, and interruptions in the flow of speech. Genetic factors have been implicated in this disorder, and previous studies of stuttering have identified linkage to markers on chromosome 12.

**METHODS—**We analyzed the chromosome 12q23.3 genomic region in consanguineous Pakistani families, some members of which had nonsyndromic stuttering and in unrelated case and control subjects from Pakistan and North America.

**RESULTS—**We identified a missense mutation in the *N*-acetylglucosamine-1-phosphate transferase gene (*GNPTAB*), which encodes the alpha and beta catalytic subunits of GlcNAc-phosphotransferase (GNPT [EC 2.7.8.15]), that was associated with stuttering in a large, consanguineous Pakistani family. This mutation occurred in the affected members of approximately 10% of Pakistani families studied, but it occurred only once in 192 chromosomes from unaffected, unrelated Pakistani control subjects and was not observed in 552 chromosomes from unaffected, unrelated North American control subjects. This and three other mutations in *GNPTAB* occurred in unrelated subjects with stuttering but not in control subjects. We also identified three mutations in the *GNPTG* gene, which encodes the gamma subunit of GNPT, in affected subjects of Asian and European descent but not in control subjects. Furthermore, we identified three mutations in the *NAGPA* gene, which encodes the so-called uncovering enzyme, in other affected subjects but not in control subjects. These genes encode enzymes that generate the mannose-6-phosphate signal, which directs a diverse group of hydrolases to the lysosome. Deficits in this system are associated with the mucolipidoses, rare lysosomal storage disorders that are most commonly associated with bone, connective tissue, and neurologic symptoms.

**CONCLUSIONS—**Susceptibility to nonsyndromic stuttering is associated with variations in genes governing lysosomal metabolism.

Stuttering, also known as stammering, is a common speech disorder that has been recognized since antiquity and affects all populations and language groups.[1] Although the underlying causes of stuttering are unknown, results of twin studies,[2–6] adoption studies,[7,8] and family studies[9,10] support a role for genetic contributions in the etiology of this disorder. Genetic-

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linkage studies have provided suggestive or significant evidence of linkage with numerous loci across the genome.[11–14] On the basis of a study involving a group of consanguineous families in Pakistan,[12] the strongest linkage is with a locus on the long arm of chromosome 12. We analyzed this locus in a group of affected Pakistani families and in a series of affected but unrelated subjects from Pakistan, North America, and Britain, as well as in unaffected, unrelated control subjects from both Pakistan and North America.

## METHODS

We analyzed families that had participated in previous linkage studies[12] and focused on the largest family, designated PKST72 (Fig. 1). In addition, we studied unrelated cases of stuttering in 46 Pakistani subjects (one from each of the previously studied families12) and 77 additional unrelated persons with stuttering from Pakistan, as well as 270 affected, unrelated persons from the United States and England. This last group was enrolled through public appeal, and potential participants were required to meet the following criteria on screening: age of 8 years or older; stuttering duration of 6 months or longer; evidence of a family history of stuttering; and speech characterized by more than 4% stuttering dysfluencies, as measured with the Stuttering Severity Instrument, 3rd edition (SSI-3),15 or a well-characterized standard reading passage.16 The mean and the overall distributions of dysfluency scores have been shown to be similar with the use of these two tests,15,16 and the distribution of scores with the use of these two instruments in our population of stuttering subjects was similar as well (see Fig. 1 in the Supplementary Appendix, available with the full text of this article at NEJM.org). Young children, in whom recovery from stuttering is common, were excluded from the study; also excluded were subjects who reported neurologic or psychiatric symptoms.

The ancestry of the Pakistani and North American–British participants was determined by self-report (see the Supplementary Appendix); all Pakistani participants were Asian in origin, and the affected North American–British participants described themselves as white (206 participants), black (25), Asian (16), Hispanic (13), Native American (1), and other or unknown (9). The mean (±SD) age in our sample of affected, unrelated subjects was 30.7±12.1 years (for the age distribution, see Fig. 2 in the Supplementary Appendix). The Pakistani control subjects consisted of 96 age-matched and sex-matched persons with normal speech from the same region of Pakistan. The control subjects consisted of 276 well-characterized, neurologically normal North American whites who were initially ascertained and evaluated as control subjects in a large study of Parkinson's disease (Coriell Institute for Medical Research).

All participants provided written informed consent, and the study was approved by institutional review boards of the National Institutes of Health and the Center of Excellence in Molecular Biology in Lahore, Pakistan.

Gene identification and bioinformatic analyses were based on the UCSC Genome Browser, March 2006 assembly. Comparative genomic hybridization[17] was performed with the use of a custom CGH 385K Array (NimbleGen), which was designed to query 10 megabase pairs centered on base pair 99,197,889 (i.e., base pair 94,220,151 to base pair 104,175,626) of the chromosome 12 sequence in seven affected and three unaffected Pakistani subjects. DNA sequencing was performed on genomic DNA that was purified with the use of standard methods. Genetic variants identified by sequencing were considered for further analysis if they met the following criteria: they had not previously been described as a common single-nucleotide polymorphism in the worldwide population (as indicated by an rs number); they altered a known functional protein motif or resulted in an amino acid insertion or deletion; they altered an amino acid that is perfectly conserved in mammals; and they produced a prominent physical alteration, such as a change in charge or polarity, in the amino acid.

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Protein-sequence alignments were performed with the use of MultAlin 5.4.1.[18] Clinical evaluations included a medical history interview and physical examination, a limited skeletal survey, an echocardiographic examination, evaluations of urine for excreted oligosaccharides and of blood for lysosomal hexosaminidase activity, and a dilated-pupil ophthalmologic examination.

## RESULTS

### LINKAGE STUDY AND GENE EVALUATION

Previous results provided maximum evidence that a marker at the *PAH* gene was linked to stuttering. [12] We sequenced the 300-kb interval centered on the *PAH* gene in five affected subjects: three from Family PKST72 (Fig. 1) and one each from two other affected Pakistani families (Family PKST51 and Family PKST54); all three families gave nonparametric linkage scores that supported linkage at this locus (data not shown). We also sequenced the same region in three unrelated Pakistani control subjects. No plausible genetic-variant candidates were detected, so we enrolled more members of Family PKST72 and performed additional two-point linkage analyses in this family, including all the genotyped family members shown in Figure 1. These analyses confirmed linkage on chromosome 12q but resulted in maximal linkage scores more proximally, with a peak at the marker D12S1607 (Fig. 3 in the Supplementary Appendix). The results indicated that the region from D12S101 to D12S1597 (extended from base pair 94,220,151 to base pair 104,175,626 in the March 2006 UCSC Genome Browser assembly) provided evidence for linkage in this family. We undertook a systematic study of this 10-Mb genomic region using a variety of methods, including comparative–genomic-hybridization microarray analysis and DNA sequencing. The microarray studies revealed no insertions, deletions, or copy-number variants greater than 210 bp associated with stuttering in our test sample, which included three affected persons from Family PKST72, two from Family PKST54, and two from Family PKST51, along with three unrelated, unaffected Pakistani subjects.

Within this 10-Mb region lie 87 known and predicted genes (UCSC Genome Browser). DNA sequencing of the exons, exon–intron boundaries, upstream regulatory regions, and 3′ untranslated regions of 45 genes residing within the region of strongest linkage (Fig. 3 and Table 2 in the Supplementary Appendix) revealed numerous coding variants in affected subjects. Segregation of these variants was first tested in Family PKST72. Because these genes all reside within the region of linkage, most of these variants cosegregated with stuttering in this family more frequently than would have been expected by chance. However, most of these variants were found at high frequency in the unaffected Pakistani population (>5%) and thus are probably nonpathogenic polymorphisms.

### MUTATIONS IN *GNPTAB*

The variant showing the highest degree of cosegregation with stuttering in Family PKST72 was a mutation (G3598A) that predicts the substitution of a lysine residue for a glutamic acid residue at position 1200 (Glu1200Lys) in GlcNAc-phosphotransferase (encoded by *GNPTAB*) (Fig. 2). With three exceptions, the affected persons in Family PKST72 carried one or two copies of this variant. Further inspection of the affected noncarriers showed that they carry common alleles at the adjacent marker D12S1607, whereas the 25 affected family members carried either one or two copies of the least common allele of D12S1607 in this population (prevalence, 0.03). This suggests that the three noncarrier affected members stutter for reasons unrelated to variation in the *GNPTAB* gene. Two members of Family PKST72 were homozygous and nine were heterozygous for the G3598A mutation but do not currently stutter. The two homozygotes were females, who are known to have a relatively high recovery rate in this disorder,[1] and we hypothesize that they represent cases of nonpenetrance. Thus, the

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G3598A variant does not display absolute segregation with stuttering in Family PKST72 under either a dominant or a recessive mode of inheritance; an additive genetic model is more consistent with the pattern of inheritance of the trait. The fact that the highest linkage scores were obtained for the *GNPTAB* G3598A variant, combined with the lack of other plausible genetic variants within the linkage interval, suggested that this variant increases the risk of stuttering when present in either one or two copies.

We then screened for this and other potential mutations in *GNPTAB* in additional affected, unrelated Pakistani persons, including 1 affected member from each of the 46 families analyzed in our previous linkage study,[12] and in 96 unaffected matched Pakistani control subjects. We also screened 270 affected, unrelated North American–British subjects and 276 unaffected North American matched control subjects. Subsequent DNA sequencing of the 21 exons, the exon–intron boundaries, and the 5′ upstream promoter region of the *GNPTAB* gene in all cases and all controls revealed that the Glu1200Lys variant occurred in affected subjects in three other Pakistani families (PKST5, PKST25, and PKST41) from our previous linkage study,[12] in one affected North American person of Asian Indian ancestry, in two affected, unrelated Pakistani subjects, and in one Pakistani control subject. This mutation thus appears to be most common in populations from the Asian subcontinent. We identified three other potential mutations in four affected, unrelated subjects, as summarized in Table 1. None of these variants were observed in the 192 chromosomes from unaffected Pakistani control subjects or in the 552 chromosomes from neurologically normal North American control subjects. Comparative analysis revealed that the normal amino acid at each of these four positions in *GNPTAB* is conserved in all vertebrates (Fig. 2), further supporting the view that these variants represent pathologic mutations.

## EVALUATION OF GENES WITHIN THE PATHWAY

We then sequenced the 11 exons, the exon–intron boundaries, and the 5′ upstream promoter region of the *GNPTG* gene, which encodes the recognition subunit of GlcNAc-phosphotransferase,[19,20] in all case subjects and all control subjects. We identified three mutations in four affected, unrelated persons (Table 1). None of these variants were present in a total of 192 chromosomes from Pakistani control subjects or in 552 chromosomes from North American control subjects. The missense mutation observed at amino acid position 74 encodes a negatively charged glutamic acid in place of the small, nonpolar amino acids (alanine and glycine) at this position in mammals and the chicken (Fig. 2). The mutation at position 688 encodes a valine in place of leucine that occurs at this position in all mammals. The third mutation was a 9-bp duplication, encoding an in-frame duplication of amino acids 5, 6, and 7.

The GlcNAc-phosphotransferase enzyme encoded by *GNPTAB/G* acts in the pathway that generates the mannose-6-phosphate targeting signal that directs enzymes to the lysosome[21] (Fig. 3). The subsequent step in this pathway is catalyzed by *N*-acetylglucosamine-1-phosphodiester alpha-*N*-acetylglucosaminidase (NAGPA, EC 3.1.4.45), also known as the uncovering enzyme (see Fig. 3 for an explanation). Sequencing the 10 exons, the exon–intron boundaries, and the 5′ upstream promoter region of *NAGPA* in all cases and all controls revealed three different mutations in six unrelated affected persons (five heterozygotes and one homozygote) (Table 1). None of these variants were present in a total of 192 unaffected Pakistani and 552 unaffected North American control chromosomes. The mutation at amino-acid position 328 encodes a cysteine in place of an arginine that is conserved in all known mammals, while the mutation at amino-acid position 84 encodes a glutamine in place of the normal histidine that occurs in humans, chimps, rats, and mice (Fig. 2). The third mutation was a 16-bp deletion that changes the penultimate amino acid from lysine to asparagine and removes the last amino acid and the stop codon, resulting in an extended open reading frame that predicts

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the addition of 112 amino acids to the carboxyl terminus of this protein (Fig. 4 in the Supplementary Appendix).

In summary, we observed mutations in *GNPTAB, GNPTG*, and *NAGPA* in 25 of 786 chromosomes from unrelated case subjects, as compared with 4 of 744 chromosomes from control subjects (chi-square = 12.476, 1 df; P = 0.0004) (Table 3c in the Supplementary Appendix).

Mutations in *GNPTAB* and *GNPTG* have been associated with the rare inherited lysosomal storage disorders mucolipidosis types II and III, respectively, [21,22] which are characterized by disorders of the joints, skeletal system, heart, liver, spleen, and motor systems and by developmental delay. Mutations in *NAGPA* have not been reported.

### CLINICAL EXAMINATIONS

Because the affected cases in our study were ascertained on the basis of having nonsyndromic stuttering, we interviewed and carried out a more detailed clinical examination of three affected persons: two of whom were heterozygous carriers of the Ala455Ser mutation in *GNPTAB*, and one of whom was homozygous for the Arg328Cys mutation in *NAGPA*. These three persons did not have the signs or symptoms typically seen in mucolipidosis types II and III.[21] None of the three subjects had a history of developmental delay, and they have had 17, 14, and 17 years of education. On physical examination, none of the three were dysmorphic or had abnormalities of the skeleton, joints, eyes, or heart. Limited skeletal surveys showed no evidence of a dysostosis multiplex. Other than stuttering, which ranged in severity from mild to moderate-to-severe, neurologic findings were within normal limits. Elevated levels of urinary oligosaccharides, which are characteristic of some lysosomal storage disorders, were not observed. In these three subjects, plasma beta-hexosaminidase values were 29.0, 9.8, and 15.0 U per liter (normal range, 10.4 to 23.0).

## DISCUSSION

Genetic studies of stuttering are complicated by the high rate of spontaneous recovery in this disorder, especially among females,[23] and by the likelihood of nongenetic and heterogeneous causes. These factors make it probable that unaffected persons can carry mutations associated with stuttering (i.e., may have nonpenetrant mutations) and that affected persons may not carry such mutations (i.e., may represent phenocopies). In addition, our findings in Family PKST72 are consistent with the possibility of assortative mating, in which preferential matings between affected persons or families can lead to stuttering caused by different mutant alleles within one large family. Three findings support a pathogenic role of these mutations in stuttering: only one of these *GNPTAB*, *GNPTG*, and *NAGPA* mutations was observed in the unaffected control subjects (i.e., in a single chromosome); all the mutations occurred at positions at which amino acid identity is conserved to a large extent across species; and all the mutations occurred within a single, well-defined metabolic pathway. Further supporting a causative role of these mutations in stuttering is the observation that although persons with mucolipidosis types II and III predominantly have skeletal, cardiac, and ocular disorders, they often have deficits in speech, particularly in expressive speech.[24–26] These deficits have been viewed as secondary to the developmental delay that is typical in these disorders, but the sparing of some intellectual functions in mucolipidosis type III25 suggests that speech deficits may be primary rather than secondary in this disorder.

There may be several reasons why the mutations we identified in *GNPTAB* and *GNPTG* result in stuttering rather than in mucolipidosis types II and III. First, mucolipidosis types II and III are generally believed to be autosomal recessive disorders, and all the unrelated affected persons in our sample, with two exceptions, were heterozygous and thus not expected to have

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either of these disorders. Another possibility is that all but one of the mutations we identified are missense rather than protein-truncation or deletion mutations, which are typically observed in mucolipidosis types II and III, and presumably have a more severe effect on protein function. None of the mutations we observed in *GNPTAB* and *GNPTG* have been identified in persons with mucolipidosis type II or type III.[27–30]

No human disorder has yet been associated with mutations in *NAGPA*. This could be viewed as surprising, given that such mutations are predicted to have an effect on many different lysosomal enzymes. Our study suggests that a primary consequence of *NAGPA* mutation is nonsyndromic, persistent developmental stuttering.

An important reason to investigate stuttering is to better understand the neural structures and functions within the brain that generate human speech, which are poorly understood. Data regarding expression of these three genes in the human brain is limited. Data for the mouse brain are available for *NAGPA* and *GNPTG*. *GNPTG* has the most localized expression in the brain, with high levels of expression in the hippocampus, hippocampal formation, and cerebellum (according to the Allen Brain Atlas31). These structures are associated with, among other things, emotion and motor function. A person's emotional state can exert a strong effect on the severity of stuttering. 1 In addition, whereas stuttering does not affect the ability to conceptualize words and sentences, it does affect the motor functions required for fluent speech. These three genes are widely expressed in many tissues in the body throughout life, and lifelong expression of these genes is consistent with the persistent nature of stuttering in our subjects.

We found *NAGPA* mutations only in persons of European descent, and these mutations occurred in 6 of the 270 affected North American–British subjects (2%). We observed mutations in *GNPTAB* and *GNPTG* in 15 of 393 affected persons who were of South Asian or European descent (4%). Persistent stuttering affects approximately 1% of the U.S. population, corresponding to about 3 million people, and an estimated 60 million people worldwide. Although our results can explain only a small fraction of cases of stuttering, these susceptibility variants are likely to be present in a large number of affected persons. Our findings also suggest that manifestations of mutations in these genes are not limited to the very small number of people who have frank symptoms of mucolipidosis type II or III. Rather, the manifestations of lysosomal targeting deficits may be present in a larger group of patients commonly encountered in medical practice.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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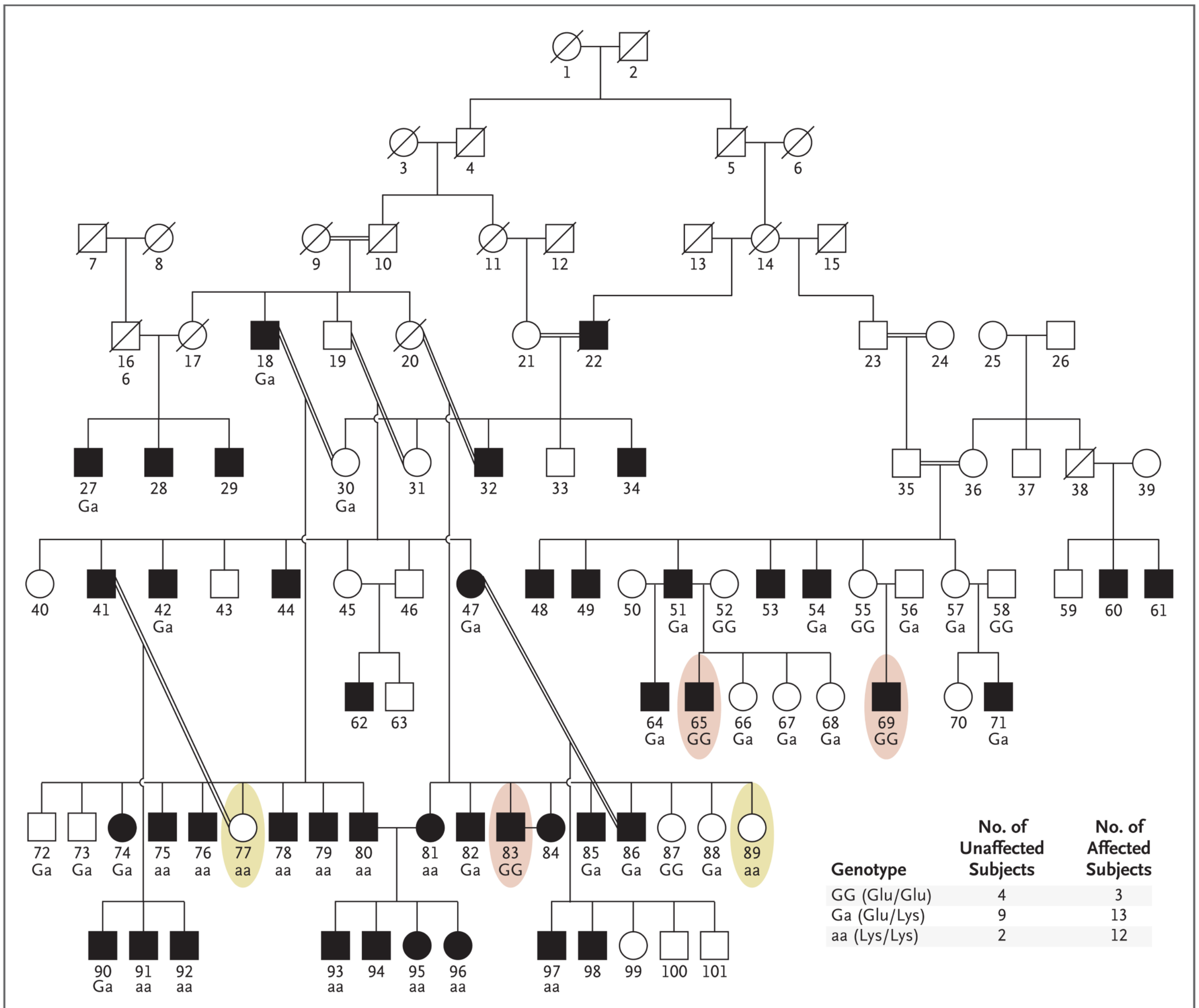


**Figure 1. Pedigree and Distribution of *GNPTAB* Glu1200Lys Mutation in Family PKST72**
Subjects 77 and 89 (green ovals) were homozygous for the mutation but were unaffected, suggesting nonpenetrance. Subjects 65, 69, and 83 (pink ovals) were affected but differed from the other affected subjects with respect to the haplotype surrounding the gene for the alpha and beta subunits of *N*-acetylglucosamine-1-phosphate transferase (*GNPTAB*). Double lines indicate consanguineous unions. Squares denote male family members, circles female members, solid symbols affected members, and slashes deceased members.



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**Figure 2. Mutations Found in *GNPTAB*, *GNPTG*, and *NAGPA* and Alignment of Their Amino Acid Sequences in Different Species**

Shaded areas indicate amino acid changes resulting from mutations in various species. In *NAGPA*, p.Phe513SerfsX113 denotes a frame-shift mutation that changes phenylalanine 513 to serine and creates a new reading frame that ends in a stop 113 amino acids downstream (see Fig. 4 in the Supplementary Appendix, available with the full text of this article at NEJM.org). *GNPTAB* denotes the *N*-acetylglucosamine-1-phosphate transferase gene encoding the subunits alpha and beta, *GNPTG* the *N*-acetylglucosamine-1-phosphate transferase gene encoding the subunit gamma, and *NAGPA* the N-acetylglucosamine-1-phosphodiester alpha-*N*-acetylglucosaminidase gene.

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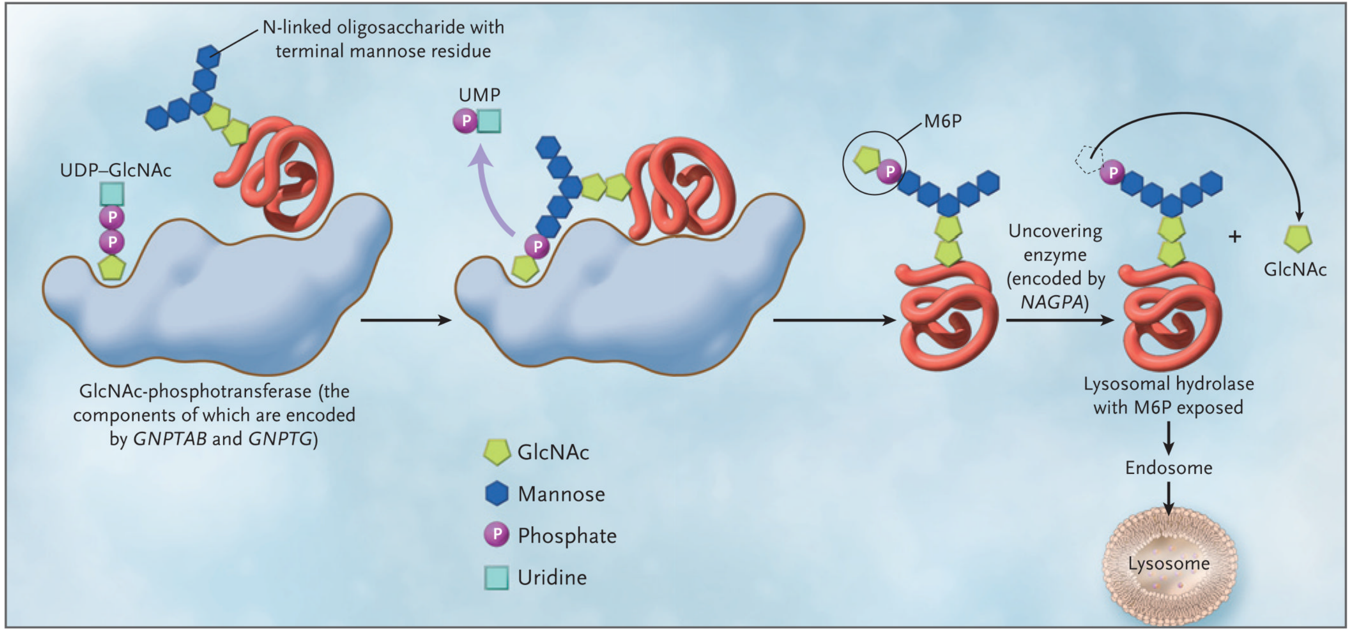


**Figure 3. Tagging for Transport of Lysosomal Enzyme**

In the first step, GlcNAc-phosphotransferase (the alpha, beta, and gamma subunits of which are encoded by the *GNPTAB* and *GNPTG* genes) catalyzes the covalent linkage of GlcNAc-1-phosphate from uridine diphosphate (UDP) to the terminal mannose residues of the N-linked oligosaccharides on enzymes destined for the lysosome. In the second step, NAGPA (*N*-acetylglucosamine-1-phosphodiester alpha-*N*-acetylglucosaminidase), also known as uncovering enzyme, removes one GlcNAc group, thereby exposing mannose-6-phosphate (M6P), which acts as the targeting signal. Enzymes with this targeting signal are then routed through the Golgi apparatus to the lysosome.

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## Table 1

Mutations Found in *GNPTAB, GNPTG*, and *NAGPA*.*

| Gene | Mutation | Change in Amino Acid | Pakistani Case Subjects (N = 123) | Pakistani Controls (N = 96) | North American–British Case Subjects (N = 270) | North American Controls (N = 276) |
|---|---|---|---|---|---|---|
| | | | *no. of mutant alleles* | | | |
| *GNPTAB* | | | | | | |
| Exon 9 | c.961A→G | p.Ser321Gly | 1 | 0 | 0 | 0 |
| Exon 11 | c.1363G→T | p.Ala455Ser | 0 | 0 | 2 | 0 |
| Exon 13 | c.1875C→G | p.Phe624Leu | 1 | 0 | 0 | 0 |
| Exon 19 | c.3598G→A | p.Glu1200Lys | 8 | 1 | 2 | 0 |
| *GNPTG* | | | | | | |
| Exon 1 | c.11_19dup | p.Leu5_Arg7dup | 0 | 0 | 1 | 0 |
| Exon 2 | c.74C→A | p.Ala25Glu | 0 | 0 | 2 | 0 |
| Exon 9 | c.688C→G | p.Leu230Val | 0 | 0 | 1 | 0 |
| *NAGPA* | | | | | | |
| Exon 2 | c.252C→G | p.His84Gln | 0 | 0 | 2 | 0 |
| Exon 6 | c.982C→T | p.Arg328Cys | 0 | 0 | 4 | 0 |
| Exon 10 | c.1538_1553del | p.Phe513SerfsX113 | 0 | 0 | 1 | 0 |

* *GNPTAB* denotes the *N*-acetylglucosamine-1-phosphate transferase gene for the alpha and beta subunits, *GNPTG* the *N*-acetylglucosamine-1-phosphate transferase gene for the gamma subunit, and *NAGPA* the *N*-acetylglucosamine-1-phosphodiester alpha-*N*-acetylglucosaminidase gene.