

ADULT-ONSET SPINOCEREBELLAR DYSFUNCTION CAUSED BY A MUTATION IN THE GENE FOR THE α -TOCOPHEROL-TRANSFER PROTEIN

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Abstract Background. Patients with isolated vitamin E deficiency have an impaired ability to incorporate α -tocopherol into lipoproteins in the liver and usually have symptoms and signs of spinocerebellar dysfunction before adolescence. Accumulated evidence suggests that the α -tocopherol-transfer protein, which is presumed to function in the intracellular transport of α -tocopherol, is abnormal in these patients.

Methods. We studied a patient from an isolated Japanese island who began to have ataxia, dysarthria, and sensory disturbances in the sixth decade of life. His serum vitamin E concentration was low (1.2 μ g per milliliter [2.8 μ mol per liter]). Exons of his gene for the α -tocopherol-transfer protein were analyzed by DNA sequencing. We also screened an additional 801 inhabitants of the island for the mutation. Both the normal and mutant α -tocopherol-transfer proteins were expressed in COS-7 cells and studied by immunoblot analysis and assay for α -tocopherol-transfer activity.

VITAMIN E is a fat-soluble antioxidant that prevents lipid oxidation in membranes. The various forms of vitamin E, such as α -, β -, γ -, and δ -tocopherol, are absorbed from the small intestine and transported in chylomicrons to the liver. In the liver, α -tocopherol, the most biologically active form of vitamin E, is incorporated into nascent very-low-density lipoproteins (VLDLs), which then enter the circulation.¹ Vitamin E deficiency is known to occur in patients with generalized fat malabsorption due to cholestatic liver disease,² abetalipoproteinemia,³ cystic fibrosis,⁴ and the short-bowel syndrome.⁵ In these patients, prolonged deficiency of vitamin E causes a decrease in the tocopherol content of nerves⁶ and results in spinocerebellar dysfunction, with progressive ataxia.⁷

In the past 14 years, 15 families have been documented worldwide as having other inherited forms of vitamin E deficiency, referred to as familial isolated vitamin E deficiency or ataxia with isolated vitamin E deficiency.⁸⁻¹⁸ These patients have normal lipid absorption and gastrointestinal function, but they have low or undetectable serum vitamin E concentrations and progressive spinocerebellar symptoms. In these patients the absorption and transport of vitamin E to the liver are normal, but hepatic incorporation of α -tocopherol into VLDLs is impaired.¹⁹ Their serum vitamin E concentrations increase when they are treated with large doses of vitamin E because of the direct transfer of

Results. The patient was homozygous for a point mutation that replaces histidine (CAT) with glutamine (CAG) at position 101 of the gene for the α -tocopherol-transfer protein. When expressed in COS-7 cells, the missense mutation produced a functionally defective α -tocopherol-transfer protein with approximately 11 percent of the transfer activity of the wild-type protein. Of the 801 island inhabitants examined, 21 were heterozygous for the His101Gln mutation. In all affected subjects, including the patient, this mutation cosegregated with an intron-sequence polymorphism. The heterozygotes were phenotypically normal and had serum vitamin E concentrations that were on average 25 percent lower than those of normal subjects (mean [\pm SD], 7.5 \pm 2.2 vs. 10.1 \pm 2.8 μ g per milliliter [17.4 \pm 5.1 vs. 23.4 \pm 6.5 μ mol per liter]; $P=0.002$).

Conclusions. α -Tocopherol-transfer protein is a determinant of serum vitamin E concentrations. An abnormality in this protein is a cause of spinocerebellar dysfunction. (N Engl J Med 1995;333:1313-8.)

α -tocopherol from chylomicrons to other circulating lipoproteins.¹⁹

The α -tocopherol-transfer protein (α -TTP) is a cytosolic liver protein with high binding affinity for α -tocopherol.²⁰⁻²³ Although its physiologic function is unclear, α -TTP purified from rat liver preferentially transfers α -tocopherol between liposomes and mitochondrial membranes in vitro.²² We recently isolated α -TTP complementary DNA (cDNA) from rats²⁴ and humans²⁵ and found that the human α -TTP gene is located on chromosome 8q (13.1-13.3), where the gene for ataxia with isolated vitamin E deficiency has been mapped.¹⁸ These observations supported the hypothesis^{15,19} that an abnormal α -TTP could cause isolated vitamin E deficiency. Recently, frame-shift mutations have been found in the α -TTP gene from families of North African and European ancestry with ataxia with isolated vitamin E deficiency.²⁶

In this report, we describe a missense mutation in the α -TTP gene of a previously described¹³ Japanese patient with delayed expression of spinocerebellar dysfunction that results in the production of a partially defective α -TTP molecule. A study of other inhabitants of the proband's native island revealed that the mutation was common and that affected heterozygotes were clinically normal but had low serum vitamin E concentrations.

METHODS

Study of the Patient and His Family

The proband was a 70-year-old man who came from a small, isolated island located 290 km from the mainland of Japan, where his family had lived for generations. He had been well until the age of 52 years, when he became aware of a feeling of unsteadiness in the dark. At the age of 57, he began to have difficulty speaking. The ataxia and dysarthria progressed very slowly. At the age of 62, he

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was found to have extremely low serum vitamin E concentrations,¹³ and his parents and children, all of whom were neurologically normal, were found to have concentrations that were low to low normal (Table 1). The low ratios of serum vitamin E to total lipids in this family indicate that the low serum vitamin E concentrations were not due to abnormal lipoprotein profiles. The low values for the vitamin E content of red cells also suggest low vitamin E content in tissues. The patient had no siblings and no family history of consanguinity.

On physical examination,¹³ his cranial-nerve function and muscle tone were normal, but his speech was slurred and scanning and he had moderate ataxia of the arms and legs. Knee and ankle reflexes were absent, and plantar responses were flexor. Sensitivity to light touch and pinprick was reduced over the fingers and toes. Position sense of his toes was lost, and vibration sense was reduced below the elbows and the iliac crests. His gait was broad-based and ataxic, and he had Romberg's sign.

He was treated with a large dose (800 mg per day) of α -tocopheryl acetate for seven months, and his serum vitamin E concentration increased to 16.0 μ g per milliliter (37.1 μ mol per liter). Romberg's sign was no longer present, and the patient's position sense and the results of nerve-conduction studies improved. He has continued to take 150 mg of α -tocopheryl acetate daily, and his neurologic dysfunction has been stable.

Population Study

We studied an additional 951 Japanese subjects to determine whether they had the same mutation in the α -TTP gene as the proband had: 801 of the approximately 10,000 inhabitants of the proband's native island and 150 subjects living in Tokyo. All subjects underwent a physical examination and routine laboratory studies. We also studied 51 residents of the island and 50 residents of Tokyo to determine the prevalence of an intron-sequence polymorphism. The study was approved by the appropriate institutional committees, and all study subjects gave informed consent.

Biochemical Analyses

Venous-blood samples were obtained from the study subjects after an overnight fast. Serum vitamin E concentrations were determined by a fluorometric method after extraction with hexane. Serum lipid concentrations were measured enzymatically.

Analysis of the α -TTP Gene

Positive clones were isolated by screening the EMBL3 human genomic DNA library with a human α -TTP cDNA probe.²⁵ Sequence analysis showed that the clones had inserts comprising the last three of the five coding exons (exons C, D, and E) and the 5' boundary of the second coding exon (exon B). On the basis of sequence data obtained from these genomic clones as well as of the known sequence of human α -TTP cDNA,²⁵ polymerase-chain-reaction (PCR) primers were synthesized to amplify DNA fragments of the α -TTP gene from the patient's leukocyte DNA. Information about the location and sequence of the primers has been deposited with the National Auxiliary Publications Service.* The patient's α -TTP coding sequence, except for the 3' ends of exons A and B, was determined by direct se-

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Table 1. Vitamin E Concentrations in the Proband and Other Family Members.*

SUBJECT	AGE (YR)/ SEX†	SERUM	SERUM TOTAL	SERUM	RATIO OF SERUM	VITAMIN E IN
		VITAMIN E	CHOLESTEROL	TRIGLYCERIDES	VITAMIN E TO TOTAL LIPIDS	RED CELLS
		μ g/ml	mg/dl		mg/g	μ g/ml of packed cells
Proband	62/M	1.2‡	206	69	0.19	0.2
Father§	87/M	5.7	—	—	—	1.2
Mother§	83/F	7.8	—	—	—	1.5
Son	36/M	5.0‡	194	105	0.81	1.2
Daughter	32/F	8.3	265	95	1.07	1.7
Normal range		5.0–15.2	130–230	50–150	>0.80	1.7–5.4

*To convert values for vitamin E to micromoles per liter, multiply by 2.32; to convert values for total cholesterol to millimoles per liter, multiply by 0.0259; to convert values for triglycerides to millimoles per liter, multiply by 0.0113.

†The ages are those at the time of examination.

‡Data were obtained from Yokota et al.¹³

§Deceased.

quencing of asymmetric PCR products with a dye-terminator sequencing kit.

In Vitro Expression of α -TTP in COS-7 Cells

The construction of a bacterial expression vector harboring human α -TTP cDNA has been described previously.²⁵ Within the wild-type construct, a *Bgl*II–*Bam*HI segment (positions 258 through 323) was replaced by the corresponding segment amplified from the patient's DNA. By the transfer of those inserts to the mammalian expression vector pRc/CMV (Invitrogen, San Diego, Calif.),²⁷ plasmid vectors encoding normal and mutant human α -TTP were constructed. Their integrity was confirmed by sequencing. COS-7 cells (8×10^6 cells in 0.5 ml of phosphate-buffered saline) were transfected with 40 μ g of each plasmid construct by electroporation. As a control experiment, the cells were transfected with 40 μ g of pRc/CMV alone (mock transfection). Cotransfection of a reporter gene showed that transfection efficiency was similar in the experiments using normal and mutant DNA. After incubation for 68 hours in 10 ml of Dulbecco's modified Eagle's medium containing 10 percent fetal-calf serum, the cells were collected in phosphate-buffered saline, disrupted by sonication, and centrifuged at 100,000 \times g for 1 hour. The supernatant was then analyzed for α -TTP. These experiments were repeated three times for each plasmid.

Immunoblot Analysis and Assay for α -TTP Activity

Immunoblot analysis and assay for α -TTP activity were performed as described previously²⁵ with some modifications. Immunoblot analysis was carried out with a polyclonal antibody raised against rat α -TTP.²⁴ The assay for α -TTP activity was performed with liposomes containing a trace amount of [³H] α -tocopherol (1.5×10^6 cpm) and nonexchangeable glycerol tri[¹⁴C]oleate. The liposomes were mixed with 0.5 mg of protein of a heavy-membrane fraction prepared from rat liver and were incubated with 0.5 mg of cell protein. The membranes were then sedimented by centrifugation, and the radioactivity of the supernatant was determined. The percentage of [³H] α -tocopherol transferred from the liposomes to the membrane fraction was calculated as described previously.²⁵

RESULTS

Identification of the α -TTP Gene Mutation

The structure of the human α -TTP gene has been partially elucidated. By direct sequencing of the fragments amplified by PCR, we determined the genomic DNA sequence coding for more than 95 percent of the amino acids of α -TTP. We found a single nucleotide alteration in the second of the five coding exons (Fig. 1).

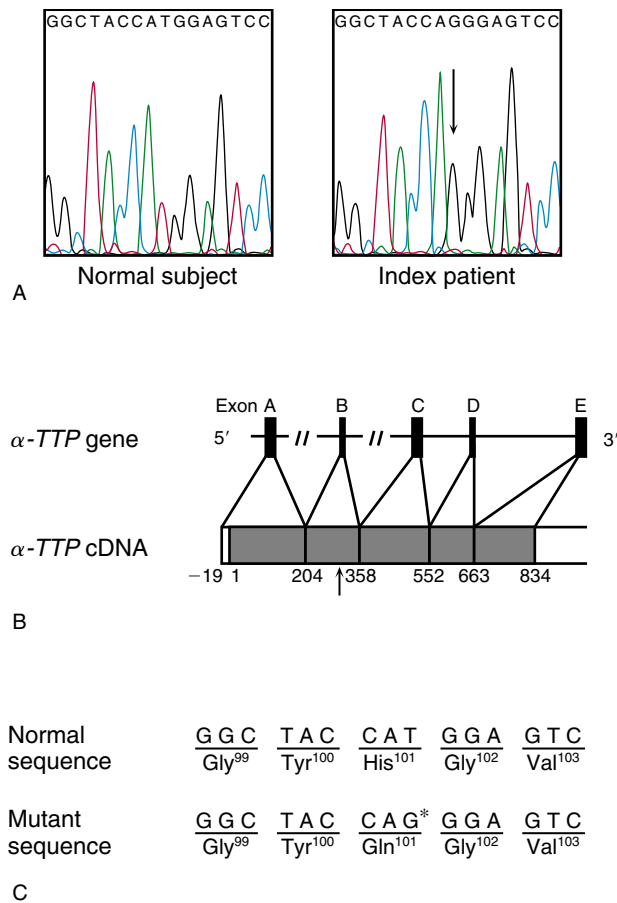


Figure 1. Identification of a His101Gln Mutation in a Patient with Neurologic Dysfunction and Low Serum Vitamin E Concentrations.

Panel A shows the partial nucleotide sequences of the sense strand of exon B of the human α -TTP gene from a normal subject and the patient. The arrow points to a guanine nucleotide substituted in the patient's gene. Panel B shows the partial structures of the human α -TTP gene and cDNA. The shaded portion of the cDNA corresponds to the region of the protein that encodes 278 amino acids. Exon boundaries are shown, with the numbers denoting the position of the last nucleotide of each 5' exon. The numbers -19, 1, and 834 denote the nucleotide positions for transcription initiation, translation initiation, and translation termination, respectively. The arrow indicates the position of the mutation. Panel C shows the partial amino acid sequences of normal and mutant human α -TTP. The guanine residue in the patient's gene that replaces the wild-type thymine residue is indicated by an asterisk.

mock transfection as well as the small measured differences in protein mass expressed had been taken into account, the mean (\pm SD) activity of the mutant α -TTP was 11.4 ± 2.2 percent of that of the wild-type α -TTP. These results indicate that the His101Gln mutation leads to the production of a functionally defective α -TTP.

Prevalence of the His101Gln Mutation among Inhabitants of the Patient's Native Island

The isolated location of the patient's homeland coupled with the absence of a family history of consanguinity led us to hypothesize that the His101Gln mutation was relatively common among the inhabitants of the island. The mutant allele was detected by digestion

The results showed that the patient was homozygous for a thymine-to-guanine (T-to-G) transversion at nucleotide 303 of the α -TTP cDNA²⁵ that replaces histidine (CAT) with glutamine (CAG) at position 101.

Functional Analysis of the Mutant α -TTP

So that the functional importance of the His101Gln mutation could be examined, expression plasmids were transfected into COS-7 cells and the cell supernatants were assayed for both the mass and activity of α -TTP. Immunoblot analysis showed that the cells expressing normal or mutant cDNA produced similar amounts of α -TTP with approximate molecular weights of 30,000 each (Fig. 2A). In contrast, the products differed significantly with respect to biologic activity, measured as the ratios of α -tocopherol-transfer activity in vitro (Fig. 2B). After the background levels of activity in the

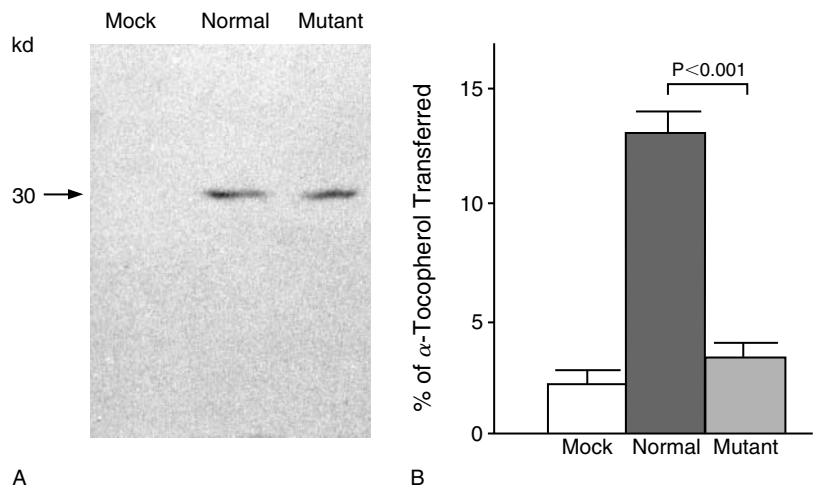


Figure 2. Effect of the His101Gln Mutation on the Expression of α -TTP in COS-7 Cells. Panel A shows a representative result of immunoblot analysis. COS-7 cells were transfected with pRc/CMV alone (Mock), pRc/CMV carrying the normal human α -TTP cDNA (Normal), or pRc/CMV carrying the mutant α -TTP cDNA with the His101Gln mutation (Mutant). Panel B shows the α -TTP activities in transfected cells. Transfer activities were measured in the supernatants of sonicated cells after centrifugation at $100,000 \times g$ for one hour in triplicate for each transfectant. Activities are expressed as the percentage of α -tocopherol transferred from the liposome (donor vesicle) to the heavy-membrane fraction (acceptor vesicle). The bars represent the mean (\pm SD) results of three independent experiments.

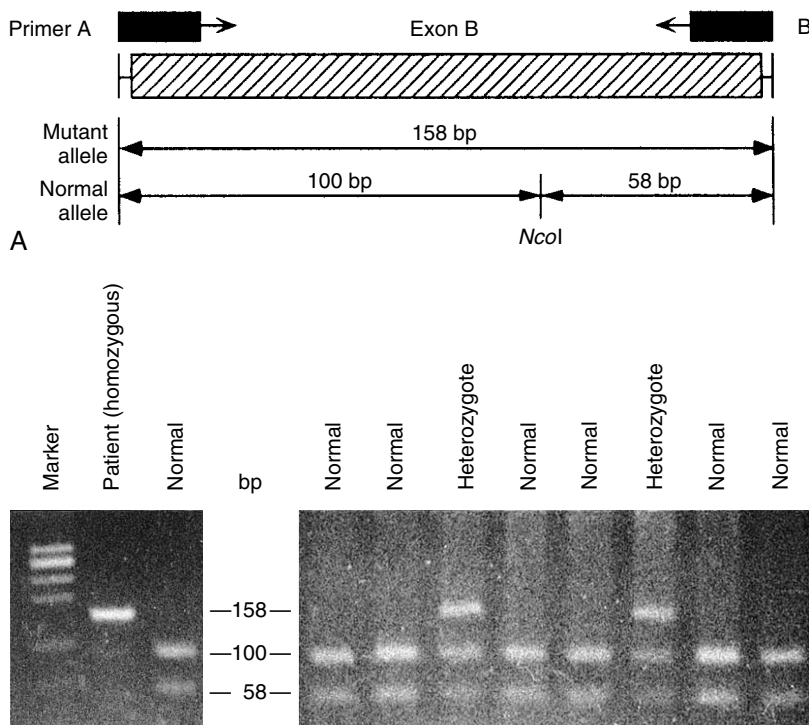


Figure 3. Detection of the His101Gln Mutation of the α -TTP Gene by Digestion with Restriction Enzyme *NcoI*.

Panel A shows the amplified portion of the human α -TTP gene. The 158-base-pair (bp) DNA fragment was amplified by PCR with primers A and B. The position of the *NcoI* site abolished by the His101Gln mutation is indicated. In Panel B, electrophoresis of digested products on a 1.5 percent agarose gel shows a homozygous subject (the index patient) and two heterozygous subjects. The molecular size marker was ϕ x174/*HaeIII*.

of DNA with restriction enzyme *NcoI*, because the His101Gln mutation disrupts a restriction site for the enzyme (Fig. 3A). The mutant allele was not detected in 150 unrelated Japanese subjects living in Tokyo. Of the 801 island inhabitants studied, however, 21 were heterozygous for the His101Gln mutation (Fig. 3B). All 21 were asymptomatic and had normal physical examinations, and none were related to the patient.

Cosegregation of the His101Gln Mutation with an Intron-Sequence Polymorphism

A sequence variation (T or C) was found in intron D of the α -TTP gene (Fig. 4). The frequencies of the T and C alleles were 0.25 and 0.75, respectively, in 50 subjects living in Tokyo, and 0.28 and 0.72, respectively, in 30 normal residents of the island. In contrast, 41 of the 42 alleles derived from the 21 subjects heterozygous for the His101Gln mutation were C alleles, and the patient was homozygous for the allele. Because the T allele in the 21 heterozygotes was derived from a normal mother homozygous for T alleles, it was evident that all of the mutant His101Gln alleles had cosegregated with C alleles. This result is consistent with the assumption that the mutant alleles originated from a common ancestor and have become prevalent in this population because of a founder effect.

Decreased Serum Vitamin E Concentrations in Subjects Heterozygous for the His101Gln Mutation

The fasting serum vitamin E concentrations of the 21 heterozygotes and 21 normal inhabitants of the island

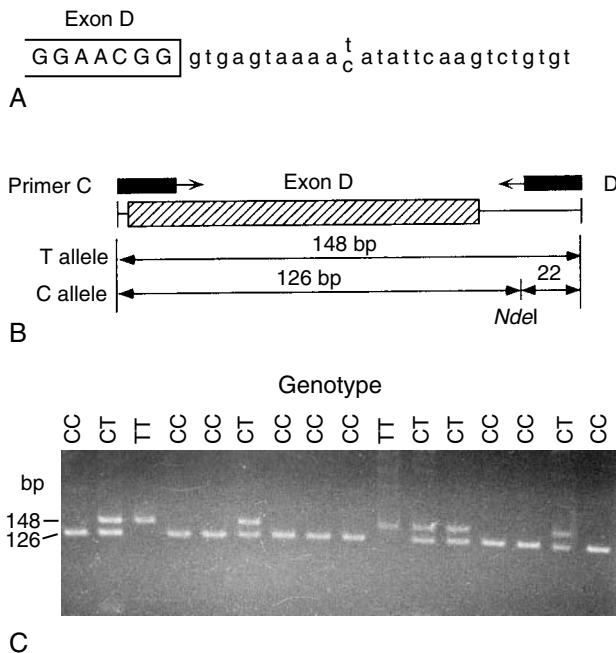


Figure 4. Detection of an Intron D Polymorphism by Digestion with Restriction Enzyme *NdeI*.

Panel A shows a cytosine-to-thymine polymorphism at a position 11 nucleotides downstream of exon D. Panel B shows a schematic diagram of the amplified portion. The 148-bp DNA fragment was amplified by PCR with primers C and D. Primer D has a sequence mismatch at its penultimate position that results in the introduction of an artificial *NdeI* site into the C allele. Panel C shows sample genotypes for the C and T alleles derived by electrophoresis on a 2.0 percent agarose gel.

Table 2. Characteristics of Heterozygotes and Normal Subjects Matched for Age, Sex, and Serum Lipid Concentrations.*

CHARACTERISTIC	HETEROZYGOTES (N = 21)	NORMAL SUBJECTS (N = 21)	P VALUE†
Age (yr)	60.7±12.3	60.5±12.2	0.96
Sex (M/F)	6/15	6/15	—
Serum total cholesterol (mg/dl)	205±28	208±26	0.70
Serum triglycerides (mg/dl)	112±66	110±62	0.89
Serum vitamin E (μ g/ml)	7.5±2.2	10.1±2.8	0.002
Intron D polymorphism (no. of T alleles/no. of C alleles)	1/41	12/30	<0.001

*Plus-minus values are means \pm SD. To convert values for total cholesterol to millimoles per liter, multiply by 0.0259; to convert values for triglycerides to millimoles per liter, multiply by 0.0113; to convert values for vitamin E to micromoles per liter, multiply by 2.32.

†All P values are two-tailed.

are shown in Table 2. The subjects were matched for age, sex, and serum cholesterol and triglyceride concentrations, because these variables affect vitamin E values.²⁸ As in the obligate heterozygotes of the patient's family (his parents and children) (Table 1), most of those heterozygous for the His101Gln mutation had

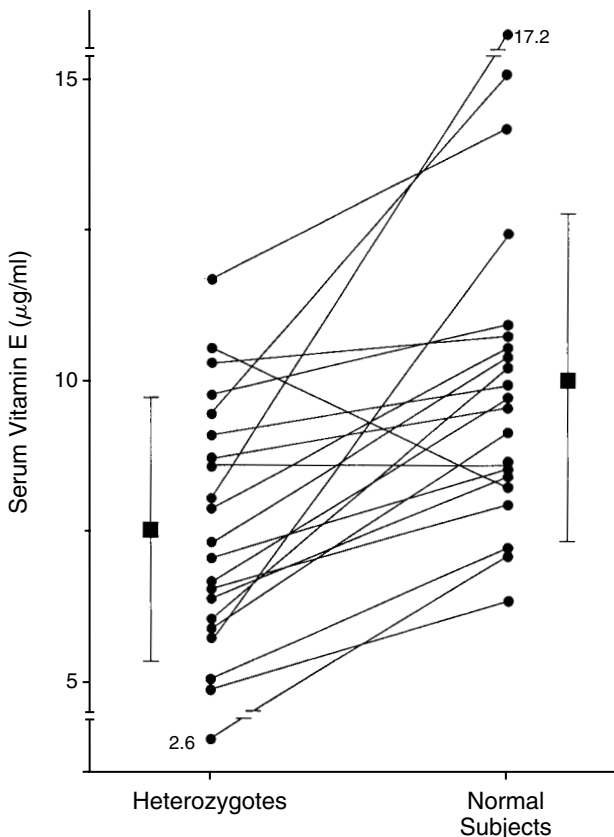


Figure 5. Serum Vitamin E Concentrations in Subjects Heterozygous for the His101Gln Mutation and Normal Subjects.

The dots representing the heterozygotes are each connected to a dot representing a normal subject matched for age, sex, and serum lipid concentrations. The mean (\pm SD) values for each group are given (squares and bars). To convert values for vitamin E to micromoles per liter, multiply by 2.32.

serum vitamin E concentrations that were low to low normal (Fig. 5).

DISCUSSION

Using genetic, biochemical, and epidemiologic approaches, we have demonstrated that a point mutation of the α -TTP gene is responsible for isolated vitamin E deficiency in a Japanese patient with spinocerebellar ataxia. Although they were not examined by an in vitro expression study in transfected cells, three frame-shift mutations of the α -TTP gene were recently found in multiple families with familial isolated vitamin E deficiency or ataxia with isolated vitamin E deficiency.²⁶ Taken together, it is clear that these disorders represent a single disease entity caused by a variety of mutations in the α -TTP gene.

Our patient was unusual with respect to the late onset of neurologic dysfunction as well as the mild phenotype.¹³ The patient began to have symptoms in his sixth decade, whereas the patients described in other reports usually had symptoms within the first two decades of life. The progression of neurologic symptoms has been slow in our patient, but was more rapid in other patients, most of whom required assistance in daily activities.^{11,12} The patient we studied was homozygous for a missense mutation that results in the production of a functionally defective α -TTP with reduced transfer activity, which is consistent with the previous observation that the patient's response to a natural stereoisomer (*RRR*- α -tocopherol) differed from that to a synthetic stereoisomer (*SRR*- α -tocopherol) and that the natural isomer could be partially incorporated into VLDL.²⁹ In contrast, the frame-shift mutations found in homozygotes with severe clinical manifestations may result in the production of less active α -TTP.²⁶ The patient's serum vitamin E concentration was also not as low as the concentrations in patients with abetalipoproteinemia and other conditions with more serious neurologic symptoms. Thus, it seems reasonable to assume that the residual transfer activity of the mutant α -TTP modified the clinical course of the disease, as shown in other diseases,³⁰ by preventing the onset of neurologic dysfunction during childhood when the nervous system is immature and more susceptible to damage by oxidative stress. The existence of this adult-onset case of the disease indicates the importance of vitamin E in maintaining the integrity of the nervous system after adolescence.

We also found that subjects who were heterozygous for the His101Gln mutation had significantly lower serum vitamin E concentrations than normal subjects, suggesting that the heterozygous state for this α -TTP mutation is a genetic determinant of serum vitamin E concentrations in vivo. In heterozygotes the effect of the His101Gln mutation on the vitamin E concentration is relatively great, because obligate heterozygotes from other affected families do not always have low vitamin E concentrations.^{12,15} Thus, in subjects who are heterozygous for the His101Gln mutation, the presence of

some functionally defective α -TTP may compromise the function of the normal α -TTP — for example, through competition for common substrates or the formation of dimers between normal and mutant α -TTP. Approximately 1 in 38 inhabitants of the patient's native island is presumed to be a heterozygote who could be anticipated to have a reduced serum vitamin E concentration throughout life. Further investigation of this population would provide a unique opportunity to examine the possible association between serum vitamin E concentrations and the incidence of diseases such as cancer.³¹

Our results serve to illustrate the protective role of vitamin E against neurologic damage and emphasize the importance of early diagnosis of isolated vitamin E deficiency, because daily supplementation with high doses of vitamin E can prevent or mitigate the associated neurologic dysfunction.

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