BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **225,** 180–185 (1996)
ARTICLE NO. 1150

A Novel Mutation in the Mitochondrial tRNA^{Thr} Gene Associated with a Mitochondrial Encephalomyopathy

Ichizo Nishino,*[,1] Ayumi Seki,† Yoshihiro Maegaki,† Kenzo Takeshita,† Satoshi Horai,‡ Ikuya Nonaka,§ and Yu-ichi Goto*

*Department of Ultrastructural Research, National Institute of Neuroscience; §Department of Laboratory Medicine, National Center Hospital for Mental, Nervous and Muscular Disorders, NCNP, Kodaira, Tokyo, Japan; †Department of Neuropediatrics, Tottori University Hospital, Tottori, Japan; and ‡Department of Human Genetics, National Institute of Genetics, Mishima, Japan

Received July 1, 1996

A novel G-to-A transition at nucleotide 15915 in mtDNA is described. The patient showed a combination of muscle weakness, hearing loss, mental retardation, and seizures. Muscle biopsy showed RRFs and focal COX deficiency. We sequenced all mtDNA, and found 5 novel nucleotide substitutions. Three of them were synonymous mutations, one was a missense mutation in cytochrome *b* gene (A → G at nt 15422), and the last one was the 15915 mutation in tRNA^{Thr} gene. We screened for the 15422 and the 15915 mutations with mismatch primers and found that one of 104 normal individuals carried the former one and none of 175 had the latter one. The 15422 mutation existed in homoplasmic states both in the patient and the normal individual, suggesting that this is a polymorphism. In constrast the 15915 mutation resided in heteroplasmic states in muscle, skin fibroblast and blood. The nucleotide substitution at nt 15915 disrupts a highly conserved base pair in anticodon stem of the tRNA^{Thr}. Our data suggest that the 15915 mutation is an additional mtDNA mutation responsible for mitochondrial encephalomyopathies.

MtDNA encodes major components of respiratory enzymes, two rRNA and 22 tRNA (1). Various point mutations in mtDNA have been associated with mitochondrial disorders (2) and most of those associated with mitochondrial encephalomyopathies are allocated in tRNA genes. Therefore only mitochondrial tRNA genes have usually been sequenced to find a new mutation in patients with mitochondrial encephalomyopathies. We, however, have occasionally found that some patients with typical myopathological features of mitochondrial encephalomyopathy including RRF have no demonstrable pathologic mutation in mitochondrial tRNA genes. Recently mutations in the region other than tRNA genes have been reported in relation to mitochondrial myopathies (3, 4).

We examined a boy who from 8 years of age had both the central nervous system and myopathic symptoms and whose muscle biopsy showed typical pathologic features of mitochondrial myopathy including RRF and COX deficiency. Screening for previously known mutations including deletions (5, 6), A-to-G mutation at nt 3243 (7), and A-to-G mutation at

[1] Correspondence: Ichizo Nishino, MD, Department of Ultrastructural Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry (NCNP), 4-1-1 Ogawahigashi-cho, Kodaira, Tokyo, 187 Japan. Fax: +81-423-46-1749. E-mail: nishino@ncnaxp.ncnp.go.jp.

Abbreviations used: bp, base pair(s); COX, cytochrome *c* oxidase; CT, computed tomography; ddNTP, dideoxyribonucleoside triphosphate; IQ, intelligence quotient; nt, nucleotide; MERRF, myoclonus epilepsy associated with ragged red fibers; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes; mtDNA, mitochondrial DNA; PCR, polymerase chain reaction; RRF, ragged-red fiber; rRNA, ribosomal RNA; SD, standard deviation; tRNA, transfer RNA.

0006-291X/96 $18.00

nt 8344 (8) were unrevealing. In order to determine whether this patient carried a mutation in mtDNA, we sequenced the entire mitochondrial genome.

PATIENT

Since the clinical and pathologic findings will be described elsewhere in details, only a brief summary is given. A 16-year-old boy was referred to Tottori University Hospital because of seizures, progressive hearing loss and muscle weakness. He had been in good health until 2 years of age when he developed generalized convulsions. He was initially placed on phenobarbital and later on valproic acid. Headaches and vomiting accompanied the convulsive attacks. He had progressive hearing loss since 9 years of age. His school performance was always at the lowest level. He developed ptosis and muscle wasting from the age of 13 years. All other family members were healthy except his mother who had cardiac arrhythmia.

On examination, he was mentally retarded with IQ of 70. His height was 141.5 cm (−4.9 SD) and weight was 28 kg (−3.4 SD). He also had hypogonadism. Predominantly proximal muscle weakness and wasting, with ptosis and sensorineural hearing loss were found. Myoclonus was occasionally seen in both upper and lower extremities. There was neither cerebellar sign nor sensory disturbance.

Laboratory examination showed high lactate levels in both serum (2.5 mmol/L) (normal: 0.5−2.0 mmol/L) and cerebrospinal fluid (3.8 mmol/L) (normal: 0.5−2.0 mmol/L). CT showed generalized cerebral atrophy and calcifications in the putamen, globus pallidus, caudate head, and dentate nucleus. Muscle biopsy revealed a moderate variation in fiber size, marked type 2B fiber atrophy, a few RRF, and focal COX deficiency. RRF were not necessarily COX deficient.

METHODS AND RESULTS

Total DNAs were isolated from frozen muscle, skin fibroblast and blood samples of the patient. For sequencing we used the DNA extracted from muscle. We designed 12 sets of primers to amplify the DNA fragments (Table 1) and ninety-nine sequence primers for both L and H strands which cover all the mtDNA. Amplified fragments by PCR were purified by MicroSpin™ S-400 HR Columns (Pharmacia Biotech), and cycle sequencing reactions were performed as indicated by the manufacturer with Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems, Perkin Elmer). Excess ddNTP labeling was removed with MicroSpin S-200 HR Columns (Pharmacia Biotech). Samples were electrophoresed through 5% Long Ranger (FMC BioProducts) gel by using a model 377 automatic DNA sequencer (Applied Biosystems, Perkin Elmer). Sequence data were compared with the previously published sequence (1) and 54 nucleotides were found to be different (Table 2). Forty nucleotides were located in the coding region and the other 14 nucleotides were in D-loop. In D-loop, conserved sequence blocks I, II, III, promoter sites, and mtTF1

TABLE 1
Twelve Primer Sets Covering the Entire mtDNA

	Forward primer	Reverse primer	Length (bp)
1	597–616	2498–2479	1902
2	1908–1927	3962–3943	2055
3	3454–3473	4970–4951	1517
4	4659–4678	6299–6280	1641
5	5874–5893	8310–8291	2473
6	7811–7830	9642–9623	1832
7	9001–9020	11335–11316	2335
8	10660–10679	12684–12665	2025
9	12315–12334	14451–14432	2137
10	13595–13614	15825–15806	2233
11	15071–15090	145–126	1646
12	16134–16153	1032–1013	1468

TABLE 2
Mutations in the Patient

Position	Change	Region	Amino acid change
750	A → G	12SrRNA	
1047	A → G	12SrRNA	
1438	A → G	12SrRNA	
2626	T → C	16SrRNA	
2706	A → G	16SrRNA	
2772	C → T	16SrRNA	
3106	del C	16SrRNA	
3423	G → T	ND1	syn
4386	T → C	tRNA-Gln	
4769	A → G	ND2	syn
4958	A → G	ND2	syn
4985	G → A	ND2	syn
6455	C → T	COI	syn
7028	C → T	COI	syn
7337*	G → A	COI	syn
8701	A → G	ATPase6	Thr → Ala
8860	A → G	ATPase6	Thr → Ala
9540	T → C	COIII	syn
9559	G → C	COIII	Arg → Pro
9824	T → C	COIII	syn
10398	A → G	ND3	Thr → Ala
10400	C → T	ND3	syn
10730*	A → G	ND4L	syn
10873	T → C	ND4	syn
11143*	C → T	ND4	syn
11335	T → C	ND4	syn
11719	G → A	ND4	syn
12705	C → T	ND5	syn
12771	G → A	ND5	syn
13702	G → C	ND5	Gly → Arg
14199	G → T	ND6	Pro → Thr
14272	G → C	ND6	Phe → Leu
14365	G → C	ND6	syn
14368	G → C	ND6	Phe → Leu
14783	T → C	Cytb	syn
15043	G → A	Cytb	syn
15301	G → A	Cytb	syn
15326	A → G	Cytb	Thr → Ala
15422*	A → G	Cytb	Ile → Val
15915*	G → A	tRNA-Thr	
16140	T → C	D-loop	
16209	T → C	D-loop	
16223	C → T	D-loop	
16262	C → T	D-loop	
16274	G → A	D-loop	
16320	C → T	D-loop	
16399	A → G	D-loop	
16519	T → C	D-loop	
73	A → G	D-loop	
146	T → C	D-loop	
263	A → G	D-loop	
303	ins C	D-loop	
311	ins C	D-loop	
489	T → C	D-loop	

*, Novel mutations in the coding regions; syn, synonymous mutation; del, deletion; ins, insertion; CO, cytochrome c oxidase; ND, NADH-ubiquinone oxidoreductase; Cytb, cytochrome b.

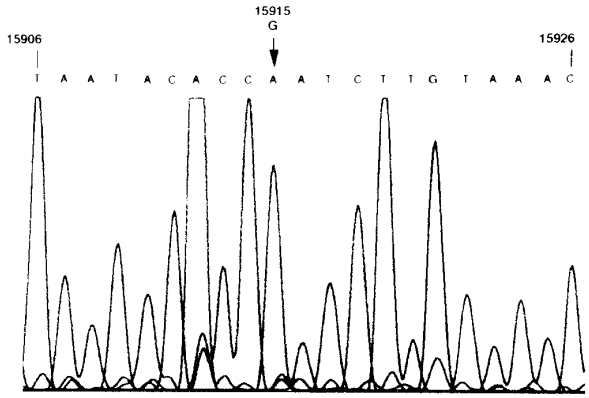

FIG. 1. Electropherogram of cycle sequence. Electrophoresed samples were analyzed with Sequence Analysis software (Applied Biosystems, Perkin Elmer). Guanine at nt 15915 was substituted for adenine.

binding sites (9) were all spared. Previous reports for polymorphism were checked in reference 10 and Mitochondrial Human Genome Database at Emory University in Atlanta (http://www.gen.emory.edu/mitomap.html) on May 14, 1996 at 10 AM GMT. Among 40 mutations in the coding region, 35 nucleotide substitutions had already been reported as polymorphism and the remaining 5 were novel. Out of 5 substitutions, G to A at nt 7333, A to G at nt 10730, and C to T at nt 11143 were all synonymous mutations in the coding regions. Only A to G substitution at nt 15422 was nonsynonymous because the nucleotide at nt 15422 is located at the first codon position changing isoleucine to valine in cytochrome *b* protein. The last transition, G to A at nt 15915 (Fig. 1), resided in the anticodon stem of $tRNA^{Thr}$ gene (Fig. 2). The nucleotides at this position are highly conserved among other species during evolution (Fig. 3).

In order to evaluate whether the 15422 and 15915 mutations were pathognomonic, we designed two mismatch primers, which created a *Dde* I (New England BioLabs) recognition site and a *Cla* I (Takara) recognition site in the mutant mtDNA, respectively. We screened placental DNA from normal individuals.

For evaluating the 15422 mutation, we used the forward primer of 5′-AATCACCTTCCACCCTTACTACTCA-3′ (mismatch sites underlined) which corresponded to nt 15379–15421 and the reverse primer corresponded to nt 15619–15600. The condition for PCR was 30 cycles of 94°C for 15 seconds, 55°C for 15 seconds, and 72°C for 30 seconds. Amplified fragments were digested with *Dde* I overnight.

Mutant mtDNA existed in a homoplasmic state in the DNA from muscle, fibroblast, and blood of our patient. We found the same substitution in one healthy individual among 104 Japanese people. Therefore it is highly likely that this substitution was a polymorphism.

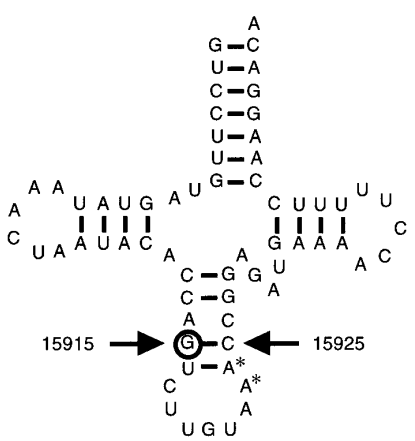

FIG. 2. Secondary structure of human mitochondrial $tRNA^{Thr}$. Guanine at 15915 is base paired with cytosine at 15925 in the anticodon stem. Asterisks indicate the nucleotides at 15923 and 15924, which have already been reported to be disease-related (13).

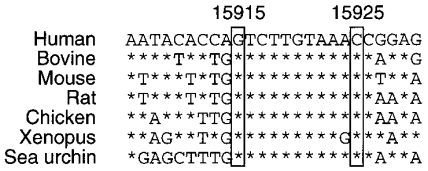

FIG. 3. Conservation of nucleotide at 15915 through evolution. Guanine at 15915 and cytosine at 15925 are well-conserved from sea urchin to human. Asterisk indicates the same nucleotide as in humans. Data used in preparing this figure were derived from the tRNA database in the EMBL data library (ftp://ftp.embl-heidelberg.de/pub/databases/trna) (12) on May 14, 1996 at 11 AM GMT.

For the 15915 mutation the forward primer corresponded to nucleotide position from 15756 to 15775 and the sequence of the reverse primer was 5′-AGGTTTTCATCTCCGGTTTAATCGA-3′ (mismatch sites underlined) which corresponded to nt 15940–15916. PCR was performed under the same condition as for the 15422 mutation and PCR products were digested with *Cla* I overnight.

In contrast to the 15422 mutation, 15915-positive mtDNA was found in heteroplasmic states in all examined tissues of the patient, *i. e.*, muscle, skin fibroblast, and blood. None of the blood samples from family members had this mutation (Fig. 4). We screened 175 normal individuals of various ethnic origins and 64 unrelated patients with two major subclasses of mitochondrial encephalomyopathies (19 patients with MERRF and 45 patients with MELAS) and none of them carried this point mutation.

To estimate the percentage of the mutant mtDNA of the 15915 mutation, we performed a ''last cycle cold PCR''. After the PCR described above, we added R110-dUTPs (Applied Biosystems, Perkin Elmer) and performed an additional cycle of 94°C for 2 minute, 55°C for 1 minute and 72°C for 12 minutes. After purification with MicroSpin S-300 HR Columns (Pharmacia Biotech) to remove excess dUTPs, PCR fragments were digested with *Cla* I under the same condition described above. Samples were electrophoresed on a 5% polyacrylamide non-denatured gel and were analyzed by a model 377 automatic DNA sequencer (Applied Biosystems, Perkin Elmer) with GeneScan software (Applied Biosystems, Perkin Elmer). The percentage of the mutant mtDNA was as follows; muscle 74%, fibroblast 32%, and blood 18%.

DISCUSSION

Among five newly found nucleotide substitutions, we did not consider three mutations at nts 7333, 10730 and 11143 to be pathologic because the corresponding amino acids are not altered. Actually, no synonymous nucleotide substitution in the coding region of mtDNA has

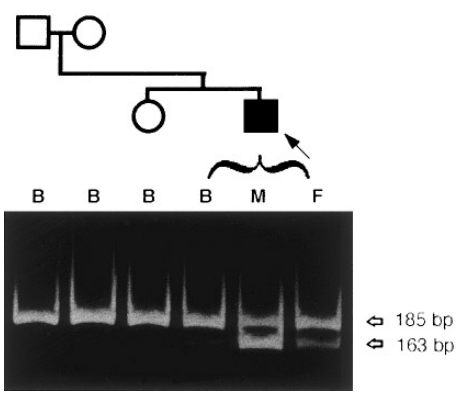

FIG. 4. The 15915 mutation: heteroplasmy in the patient and screening for family members. PCR was performed with a mismatch primer which created *Cla* I recognition site in mutants. Amplified fragments, were digested with *Cla* I, electrophoresed through 8% polyacrylamide gel, and stained with SYBR™ Green I (Molecular Probes). When the 185 bp PCR products contained the 15915 mutation, they were cleaved into 22 bp (not visible) and 163 bp fragments. Only the proband had this mutation and the mutant and the wild type DNA coexisted in all tissue examined (heteroplasmy). B, M, and F indicate blood, muscle, and fibroblast, respectively.

been associated with mitochondrial diseases. The 15422 mutation was not pathologic, either, because the mutant mtDNA existed in a homoplasmic state and one out of 104 normal subjects had this mutation. In addition, the amino acid change from isoleucine to valine due to the A to G substitution in the first codon position is one of the most frequent neutral nucleotide substitutions in human mtDNA, presumably because this amino acid change seems to be conservative in terms of chemical properties (11). Absence of discernible signs and symptoms in the previous generations of our patient or the normal subject suggests that this mutation is a neutral polymorphism. On the other hand, the 15915 mutation was not found in a large number of people and was detected in a heteroplasmic state in our patient. Thus, we assume that only the 15915 mutation is associated with this disease.

Yoon et al. reported two point mutations in the mitochondrial $tRNA^{Thr}$ gene, A-to-G transition at nt 15923 and A-to-G transition at nt 15924, in association with a fatal infantile mitochondrial disease (12). Although the clinical manifestations of our patient is definitely different from their patient, the 15915 mutation is thought to be the third disease-related mutation in $tRNA^{Thr}$ associated with mitochondrial diseases.

Normally guanine nucleotide at 15915 is base-paired with cytosine at 15925 in the anticodon stem. This G-C pairing at this position is highly conserved from sea urchin to human in evolution (13). Thus nucleotide change at this position disrupts a highly conserved hydrogen bond in the anticodon stem, which possibly makes the anticodon stem unstable. This structural alteration in tRNA might impair the function of $tRNA^{Thr}$ and influence the efficiency of translation in mitochondria, as seen in the case of the 8344 mutation (14).

Although study using rho^{o} transformants is under way, further studies are needed to clarify the functional effects of this mutation at RNA and protein levels.

ACKNOWLEDGMENT

Authors are grateful to S. M. Sumi, Department of Pathology, University of Washington, for his kind advice and suggestions.

REFERENCES

1. Anderson, S., Bankier, A. T., Barrell, B. G., de Bruijn, M. H. L., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Roe, B. A., Sanger, F., Schreier, P. H., Smith, A. J. H., Staden, R., and Young, I. G. (1981) *Nature* **290,** 457–465.
2. Mitochondrial encephalopathies: Gene mutation (1996) *Neuromusc. Disord.* **6**(2), XI–XIV.
3. Manfredi, G., Schon, E. A., Moraes, C. T., Bonilla, E., Berry, G. T., Sladky, J. T., and DiMauro, S. (1995) *Neuromusc. Disord.* **5,** 391–398.
4. Keightley, J. A., Hoffbuhr, K. C., Burton, M. D., Salas, V. M., Johnston, W. S. W., Penn, A. M. W., Buist, N. R. M., and Kennaway, N. G. (1996) *Nature Genet.* **12,** 410–416.
5. Holt, I. J., Harding, A. E., and Morgan-Hughes, J. A. (1988) *Nature* **331,** 717–719.
6. Zeviani, M., Servidei, S., Gellera, C., Bertini, E., DiMauro, S., and DiDonato, S. (1989) *Nature* **339,** 309–311.
7. Goto, Y., Nonaka, I., and Horai, S. (1990) *Nature* **348,** 651–653.
8. Shoffner, J. M., Lott, M. T., Lezza, A. M. S., Seibel, P., Ballinger, S. W., and Wallace, D. C. (1990) *Cell* **61,** 931–937.
9. Clayton, D. A. (1991) *Annu. Rev. Cell Biol.* **7,** 453–78.
10. Ozawa, T., Tanaka, M., Sugiyama, S., Ino, H., Ohno, K., Hattori, K., Ohbayashi, T., Ito, T., Deguchi, H., Kawamura, K., Nakane, Y., and Hashiba, K. (1991) *Biochem. Biophys. Res. Commun.* **177,** 518–525.
11. Horai, S., Hayasaka, K., Kondo, R., Tsugane, K., and Takahata, N. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 532–536.
12. Yoon, K. L., Aprille, J. R., and Ernst, S. G. (1991) *Biochem. Biophys. Res. Commun.* **176,** 1112–1115.
13. Steinberg, S., Misch, A., and Sprinzi, M. (1993) *Nucleic Acids Res.* **21,** 3011–3015.
14. Enriquez, J. A., Chomyn, A., and Attardi, G. (1995) *Nature Genet.* **10,** 47–55.