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A Novel Mitochondrial DNA Point Mutation in the tRNA^{Ile} Gene: Studies in a Patient Presenting with Chronic Progressive External Ophthalmoplegia and Multiple Sclerosis

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We report a new mutation, a G to A transition at nucleotide position 4298 within the mitochondrial tRNA^{Ile} gene in a patient with chronic progressive external ophthalmoplegia and multiple sclerosis. The mutation, which alters an evolutionary conserved nucleotide within the anticodon stem, was heteroplasmic in skeletal muscle but was not present in the patient's blood. Single fibre PCR analysis revealed significantly higher levels of the G4298A mutation in cytochrome *c* oxidase (COX) negative fibres than in COX-positive fibres. This mutation represents the seventh pathogenic nucleotide substitution to be found in this gene and as such confirms the tRNA^{Ile} gene as a susceptible "hot spot" for mitochondrial DNA point mutations. Of particular interest is that this patient has the clinical features of both multiple sclerosis and a mitochondrial DNA disorder.

Mitochondrial cytopathies are a heterogeneous group of clinical disorders which present with a spectrum of neurological symptoms and signs (1). The molecular genetic defect in many of these patients involves mitochondrial DNA (mtDNA) and may take the form of large-scale rearrangements of the genome (deletions and duplications) or point mutations within either protein-encoding genes or more commonly, tRNA genes. To date, some 50 pathogenic mtDNA point mutations have been described in the literature, each shown to exhibit clear association with human disease (2). Whilst different mitochondrial tRNA mutations may give rise to very differing phenotypes, no one disease phenotype is specific for any one pathogenic tRNA mutation.

We report the finding, by direct sequence analysis of the 22 mitochondrial tRNA genes, of a novel point mutation in the tRNA^{Ile} gene of a patient presenting with a severe eye movement disorder in whom a diagnosis of mitochondrial disease was suspected. The mutation was heteroplasmic in skeletal muscle, present in high levels in individual cytochrome *c* oxidase negative muscle fibres and fulfils all the accepted criteria for assigning pathogenicity.

CASE REPORT

A 49 year old woman presented with a 2 year history of left-sided facial pain typical of trigeminal neuralgia, and fluctuating parasthesia of the left hand. Examination revealed left sided optic atrophy and gaze evoked horizontal nystagmus, as well as bilateral ptosis and restriction of eye movements in all directions of gaze. On clinical grounds it was thought that this lady had multiple sclerosis, but there was some concern about the degree of ophthalmoparesis. Cranial MRI revealed multiple plaques typical of multiple sclerosis. The CSF examination contained 4 white cells/mm^3, a normal glucose and an elevated protein (0.43g/L), 22% of which was IgG. Unmatched oligoclonal bands were present in her CSF. Whilst both clinical and laboratory findings suggested that this lady had multiple sclerosis, the ophthalmoparesis was, however, atypical and her serum CK was marginally elevated (214U/L, normal $<$ 140U/L). She therefore underwent a needle muscle biopsy to investigate the possibility of a mitochondrial disorder.

METHODS

Biochemistry. Skeletal muscle was obtained from vastus lateralis by fine needle muscle biopsy using local anaesthetic. Histochemical analysis was performed as previously described (3). The activities of the individual respiratory chain complexes and ci-

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trate synthase were determined spectrophotometrically in a post-600g supernatant (4).

Molecular genetic analysis. Total DNA was isolated from skeletal muscle, blood, hair roots and buccal epithelial cells using standard protocols. Long-range PCR and Southern analysis of the skeletal muscle DNA were used to investigate the presence of rearranged mtDNA molecules, and the sample was screened for the A3243G and A8344G mutations. The 22 tRNA genes of the mitochondrial genome were amplified using M13-tailed PCR primers, and sequenced directly using dye primer chemistries on an ABI 373 automated DNA sequencer (Applied Biosystems).

Quantification of mutant mtDNA. The level of mutated mtDNA in various tissue samples was determined using last hot cycle PCR and restriction digest analysis. A 173 bp fragment containing the region of interest was amplified using the forward primer 5′ GTA-CGACCAACTCATACACC 3′ (nucleotide position 4149-4168) and the reverse mismatch primer 5′ GGGGTTTAAGCTCCTATTAT**G**T 3′ (nucleotide position 4321-4300), with the mismatch nucleotide shown in bold. Samples were subjected to 30 cycles of amplification using the following PCR conditions: denaturation at 94°C for 1 min, annealing at 57°C for 1 min, extension at 72°C for 1 min; the final extension proceeded for 8 min. Following the addition of a further 30 pmol of each primer, 1U of thermostable DNA polymerase and

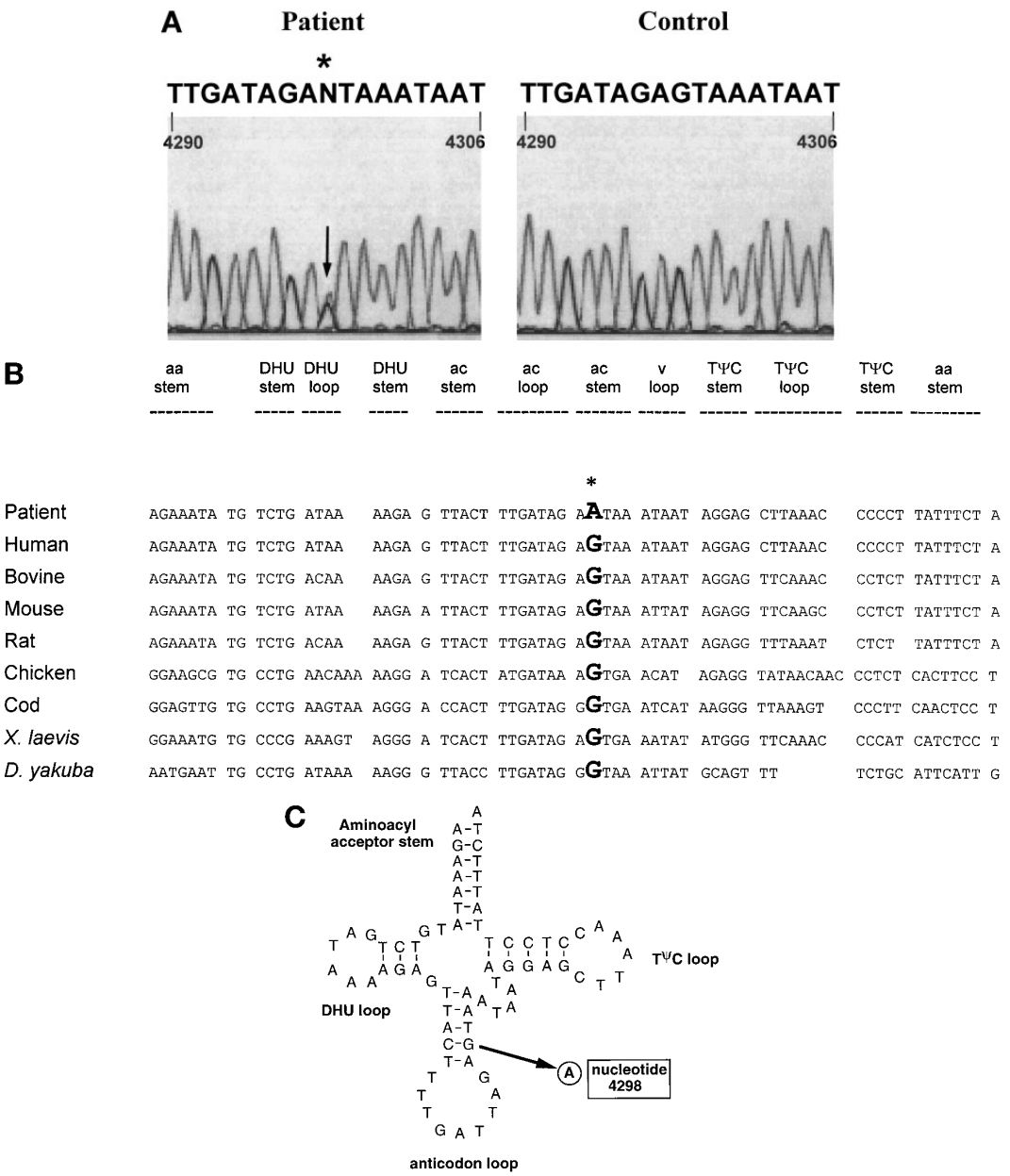

FIG. 1. Identification of a novel mutation. A. Sequence analysis of the $tRNA^{Ile}$ gene in the patient and control skeletal muscle highlighting the G to A transition at nucleotide position 4298 (shown by arrow). **B.** Nucleotide sequence comparison of $tRNA^{Ile}$ gene in different species. The G4298A transition (asterisk) within the anticodon stem changes a highly conserved residue. **C.** Schematic of $tRNA^{Ile}$ structure indicating the base pair which is disrupted by the mutation.

5μCi [α-^{32}P]-dCTP (3000Ci/mmol), reaction products were subjected to a single "hot" last cycle (94°C for 8 min, 57°C for 2 min and 72°C for 12 min). Samples were phenol/chloroform extracted and digested overnight with 10U *Rsa I*. Digested products were electrophoresed through a 12% non-denaturing polyacrylamide gel, and the radioactivity in each fragment quantified using ImageQuant software (Molecular Dynamics). The mismatch base in the reverse primer creates a *Rsa I* site in the PCR product which cleaves a 173 bp fragment to 151 bp and 22 bp respectively. This *Rsa I* site is destroyed in the presence of the G4298A mutation.

Single fibre analysis. Transverse sections (30μm) were reacted for both cytochrome *c* oxidase and succinate dehydrogenase to identify COX-positive and COX-negative fibres (3). These were isolated as previously described (5) and individual fibres placed in a PCR cocktail supplemented with 1% Triton X-100 (v/v). Samples were heated to 94°C for 5 min and PCR (30 cycles) initiated by the addition of thermostable DNA polymerase. The level of mutated mtDNA in individual fibres was determined as detailed above.

RESULTS

Muscle Histochemistry

The muscle biopsy revealed an increased variability of fibre size, ranging from 15-120μm (normal range 30-70μm). Of these fibres, 10% exhibited the classical "ragged-red" appearance, characteristic of mitochondrial disease. Subsequent reaction for cytochrome *c* oxidase activity showed that 20% of the fibres throughout the biopsy were cytochrome *c* oxidase negative, thus confirming a mitochondrial myopathy.

Activity of Respiratory Chain Complexes

The activities of complex I and complex IV were markedly decreased, at 60% and 40% of control values respectively (data not shown).

Identification of Novel tRNA^{Ile} Mutation

Southern and long-range PCR analysis failed to reveal any large-scale rearrangements of the mitochondrial genome. A screen for the A3243G tRNA$^{Leu(UUR)}$ and A8344G tRNA^{Lys} mutations did not identify any mutations. Direct sequencing of the twenty-two mitochondrial tRNA genes, however, revealed a single base change from the Cambridge sequence (6), a G to A transition at nucleotide position 4298 within the tRNA^{Ile} gene (Figure 1A). The mutation was heteroplasmic in skeletal muscle and alters a highly conserved base in the anticodon stem of the tRNA molecule (Figure 1B and 1C).

Subsequent RFLP analysis confirmed that the G4298A mutation was present in a skeletal muscle homogenate at a level of 56%, although absent in the patient's blood (Figure 2). The mutation was also present, albeit at much lower levels, in hair roots (4%) and buccal epithelial cells (9%).

Single fibre PCR showed that the level of mutant mtDNA was significantly higher in COX-negative fibres, with an average level of mutant mtDNA of 78%

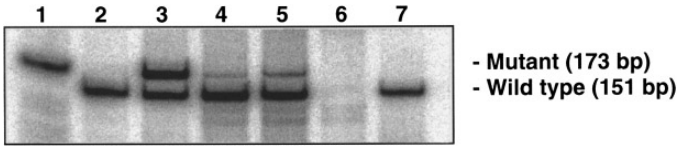

FIG. 2. Quantification of the G4298A mutation by RFLP analysis and last hot cycle PCR. **Lane 1:** uncut sample. **Lane 2:** control DNA sample. **Lane 3:** skeletal muscle. **Lane 4:** hair roots. **Lane 5:** buccal epithelia. **Lane 6:** no DNA. **Lane 7:** control DNA sample.

(range 59-93% (n=12)) than COX-positive fibres (average level of 27%, range 11-48% (n=12)) (Figure 3).

DISCUSSION

We have identified a novel base substitution at nucleotide position 4298 in the mitochondrial tRNA^{Ile} gene of a patient with optic atrophy and an eye movement disorder. This particular base change, which has not previously been described, satisfies all the current criteria used to assign pathogenicity. First, the G4298A substitution is heteroplasmic. Second, it is present at a higher level in skeletal muscle, a post-mitotic tissue, than in tissues that are rapidly dividing (blood, hair, buccal epithelia), presumably because cells with high levels of mutation that are rapidly turning over are at a selective disadvantage. Third, the mutation segregates with cytochrome *c* oxidase negative fibres within muscle. Fourth, the G-to-A transition alters a highly conserved base pair within the anticodon stem of the tRNA, potentially altering the tertiary structure of the molecule and thus its function during intramitochondrial protein synthesis. Mutations within this domain of the anticodon stem of other mitochondrial tRNA genes have been shown to cause disease 7, 8). Finally, several other disease-specific mutations have also been identified in the tRNA^{Ile} gene, associated with cardiomyopathy (9-12) or progressive external ophthalmoplegia (13, 14). Only the tRNA$^{Leu(UUR)}$ gene has more documented examples of pathogenic mutations (2), thus confirming the tRNA^{Ile} gene as another "hot-spot" for disease.

Extensive mtDNA analysis of the patient's immediate family was not possible, since they declined further investigations. This makes it difficult to make any correlations relating genotype to clinical phenotype. The patient's mother is well and has no clinical evidence of neurological disease. The patient also has two daughters in their early twenties, both of whom appear unaffected. It is therefore impossible to determine whether the mutation has been transmitted through this family, or indeed when the initial mutational event occurred.

Perhaps the most intriguing finding in this patient is the unequivocal diagnosis of multiple sclerosis (MS) in association with a pathogenic mtDNA mutation. This is of considerable interest for a number of reasons.

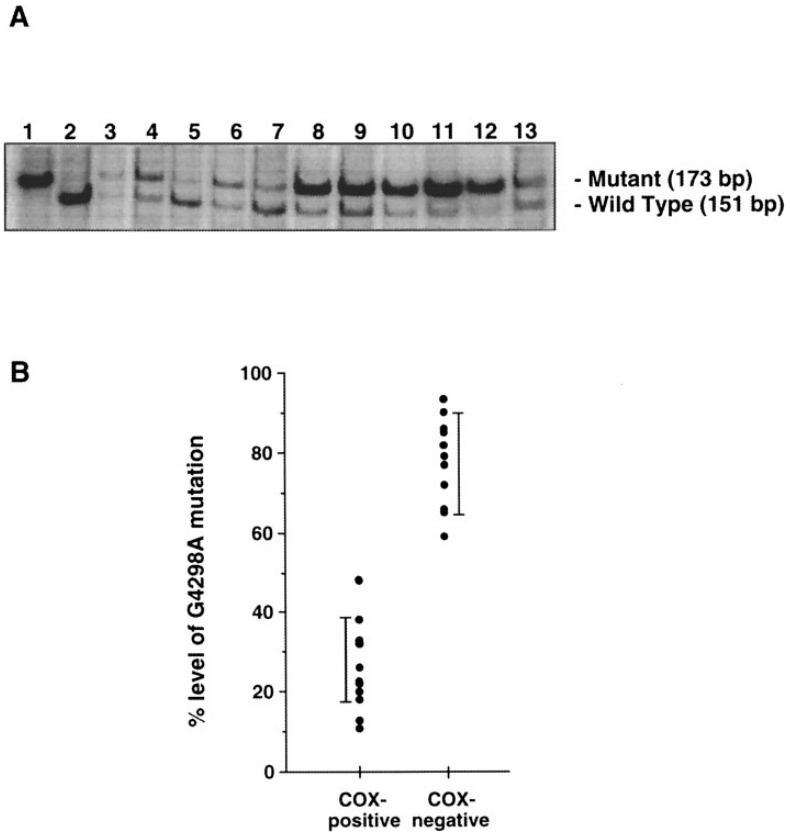

FIG. 3. Distribution of the G4298A mutation in single muscle fibres. **A.** Representative RFLP analysis of five COX-positive and five COX-negative fibres showing higher levels of mutant mtDNA are present in COX-negative fibres. **Lane 1:** uncut sample. **Lane 2:** control DNA sample. **Lanes 3-7:** COX-positive fibres. **Lanes 8-12:** COX-negative fibres. **Lane 13:** skeletal muscle. **B.** Level of mutation in twelve COX-positive fibres (average level of mutant mtDNA of 27%) and twelve COX-negative fibres (average level of mutant mtDNA of 78%). The error bars illustrate the mean (± SD) level of the G4298A mutation for each data set.

It has been hypothesised that maternally inherited mitochondrial genes may contribute to disease susceptibility in MS, as there is an excessive female transmission of MS to offspring (15). Moreover, some patients with a family history of Leber's hereditary optic neuropathy develop an illness indistinguishable from MS (16). Several of these patients studied by Harding et al., all of them women, had the HLA-DR2 antigen which is known to strongly associate with MS in north European populations (17). Whilst this suggested a possible causal link between mtDNA abnormalities and MS, several studies have failed to show any increase in the incidence of mtDNA mutations of primary pathogenic significance in the MS population (18–21).

However, our patient has both MS and a pathogenic mtDNA tRNA mutation. This raises again the possibility of a link between MS and mitochondrial disease. The occurrence of these two disorders could of course be a chance event. Alternatively, the G4298A mutation may be causing the production of an abnormal antigen which causes the MS in later life. This is possible since heteroplasmic mtDNA mutations have been shown to accumulate over time in post-mitotic tissues (5). If during childhood the level of mutation was below the critical threshold required to cause a defect, it would not express itself phenotypically. It is possible that the mutant mtDNA accumulates with age to a degree whereby it causes a biochemical defect and triggers the production of a novel antigen.

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