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Refinement of the adPEO linked locus on Chr10 and analysis of MRS4 and three other candidate genes

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Mitochondrial dysfunction is implicated in a wide variety of neurodegenerative disorders. The clinical features of mitochondrial disorders are extremely variable, but often affect heart and/or skeletal muscles and the central nervous system. In humans, the 16 569-bp, maternally inherited mitochondrial DNA (mtDNA) encodes 13 subunits of the electron transport chain, 22 tRNAs, and 2 rRNAs. However, nuclear DNA (nDNA) encodes the large majority of mitochondrial proteins. Thus, normal function of the human mitochondrion involves a highly complex interaction between the nuclear and mitochondrial genomes.

Mitochondrial diseases can therefore be a result of mutations in mitochondrial or nuclear encoded genes. Disorders caused by point mutations in the mtDNA include MELAS, MERRF, NARP and LHON, while large-scale rearrangements of mtDNA are the molecular features in Pearson's or Kearns–Sayre syndromes. Mitochondrial diseases caused by nuclear defects include COX deficiency due to mutations in the SURF-1 gene, Friedreich Ataxia, and spastic paraplegia. Finally, there is a group of diseases where the defect is in a nuclear gene, but affecting mtDNA maintenance, thereby resulting in mitochondrial dysfunction. This group of diseases is associated with mtDNA depletion or multiple mtDNA deletions. Mitochondrial diseases due to multiple mtDNA deletions are genetically and clinically heterogeneous: autosomal dominant and recessive forms and distinct clinical syndromes have been described (http://www.ncbi.nlm.nih.gov/Omim).

The autosomal dominant form usually presents with adult-onset progressive external ophthalmoplegia (adPEO) and myopathy. Deafness and cataract are also common features of this syndrome, while depression is a prominent feature in one pedigree [1]. Tremor and levodopa responsive Parkinsonism, and a severe sensory ataxic axonal neuropathy have been reported in some pedigrees (http://www.ncbi.nlm.nih.gov/Omim). The multiple mtDNA deletions are most abundant in muscle, but have also been detected at lower levels in other autopsied post-mitotic tissues.

The Mendelian inheritance pattern implies the involvement of nuclear genes in adPEO and adPEO has been mapped to two different loci. Suomalainen et al. [2] assigned the first disease locus to 10q23.3–24.3 in a large Finnish family. The region was narrowed to 7 cM when a Pakistani family was also shown to map to this region [3]. A second locus mapping to 4q34–35 was reported [4] and recently, the heart/skeletal muscle isoform of the adenine nucleotide translocator (ANT1) gene was identified as the causative gene at this locus [5]. The adPEO gene on chromosome 10 has not yet been identified. A locus had previously been mapped in three Italian families to 3p14.1–21.2 but it has since been withdrawn [5].

We have identified another family with adPEO linked to chromosome 10. Fourteen members were examined and 10 were found to be affected with varying degrees of ptosis, external ophthalmoplegia, proximal weakness and bulbar weakness. By history, another six family members are similarly affected. Muscle biopsies in two affected patients, the proband and her paternal cousin, showed patchy COX deficiency and 'ragged-red' fibres. In both patients, Southern blot analysis of *Pvu*II-digested (i.e. linearised) mtDNA from muscle, probed with a mtDNA probe revealed multiple mtDNA deletions. Respiratory chain enzyme assays showed a mild–moderate reduction of activities of those subunits containing mtDNA-encoded subunits, with normal or higher activity of the nucleus-encoded complex II. This is similar to the previously reported biochemical findings in adPEO [1].

Linkage analysis suggested linkage to the locus at 10q23.3–24.3. Fine mapping of recombination sites in the family with a further 10 markers (D10S541, D10S583, D10S198, D10S603, D10S1266, D10S1268, D10S1738, D10S540, D10S530, D10S566) defined the critical region (approximately 13 cM) to be between markers D10S1686 and D10S1738. Further details on the haplotype for this pedigree are located online (http://www.elsevier.nl/PII/s0014579301026126). Suomalainen et al. [2] defined their critical region to a 20 cM region between markers D10S198 and D10S562, while Li et al. [3] defined their region to a 10-cM region between markers D10S1709 and D10S1795. These results suggest that the causative gene for adPEO is located in the approximately 3-cM interval between D10S198 and D10S1738 where the three critical regions overlap (see Fig. 1A).

Primary candidate genes were selected from known genes mapped to our critical region. They were chosen either because they had a known function in the mitochondria or that they had mutant yeast homologues with a defective mitochondrial phenotype. We found no cDNA mutations or difference in expression levels, where investigated, in COX15, NADH: ubiquinone oxidoreductase ASH1 subunit (NDFUB8) or stearoyl-CoA desaturase (SCD). We therefore concluded that combined with the expected function of these gene products neither COX15, NDUFB8, nor SCD are likely to be the causative gene for adPEO. A fourth candidate gene, mitochondrial RNA splicing 4 (MRS4) gene, was originally described in the yeast *Saccharomyces cerevisiae*, and its mutant phenotype in yeast made it the most likely candidate gene for adPEO [6]. Yeast MRS4 is 305 amino acid residues long and belongs to a family of mitochondrial carrier proteins with similar tripartite structures.

The gene structure and function of the human MRS4 homologue was unknown, but a search of EST databases indicated that the protein is widely conserved and expressed in a variety of human and other vertebrate tissues, including adult human muscle and brain. By comparison of the cDNA sequence with a fully sequenced genomic BAC clone (NCBI High-Throughput Genome Sequence, clone RP11-85A1), we elucidated the gene structure. The human MRS4 gene covers

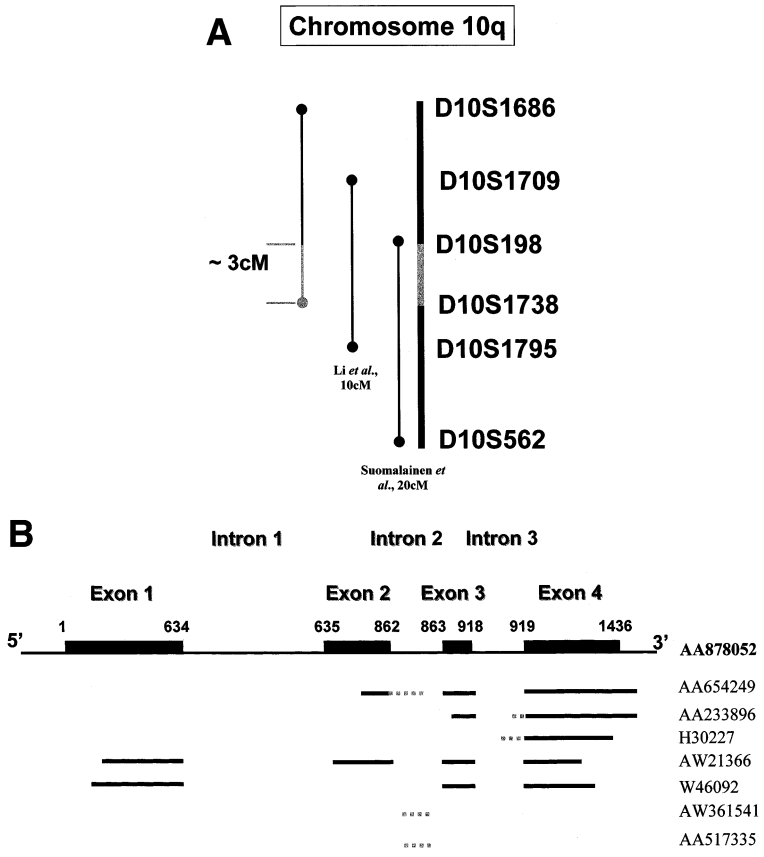

Fig. 1. A: Linkage map of adPEO region on 10q23.4-24.3. A map of the critical regions in the three studies and the overlapping interval. The order of the markers is according to the Genome Database (http://www.gdb.org.au). B: Human and mouse MRS4 transcripts. All these transcripts were found at the NCBI dbEST. Human EST's: AA878052, AA654249, AA233896, H30227, AW361541. Mouse EST'S: AW21366, W46092, AA517335. ■ exonic sequence, ▨ intronic sequence.

approximately 10 kb, contains four exons and three introns and predicts a protein of 364 amino acid residues in length. It has an extended N-terminus region when compared with other MRS4 protein sequences, the significance of this is not known. This basic structure corroborates that reported in [7].

An unusual feature of MRS4 gene expression is the many alternatively spliced mRNAs we found on analysing human, mouse, bovine, pig, and rat cDNA clones in silico (Fig. 1B). In fact, the majority of MRS4 cDNA clones that we have sequenced, or for which cDNA sequence is available from the I.M.A.G.E. consortium's expressed sequence tag (EST) database are splice variants. Many of these have long in-frame reading frames and can potentially code for MRS4-like proteins. Only one EST (AA878052, IMAGE 147912, which we have called the consensus) appeared to be a full-length clone, which we sequenced in its entirety on both strands (Fig. 1B). The remainder did not contain the 5′ end but incorporated portions of intron 2, intron 3, or both. We amplified some of these variants from patient and control muscle cDNA (data not shown). No variants of intron 1 or alternative splicing in this intron were noted. We sequenced the full 1.8-kb cDNA from both patient and control muscle and no mutations were found. Real-time PCR analysis of MRS4 RNA expression, relative to citrate synthase RNA, in muscles also did not show any significant difference between patient and two normal quadriceps muscle samples.

Prediction of donor and acceptor sites in the consensus MRS4 by analysis of the RP11-85A1 sequence (http://www.hgsc.bcm.tmc.edu/SearchLauncher/), showed that only the donor and acceptor sites used in intron 1 were recognised as functional. The donor and acceptor sites used in intron 2 and intron 3 respectively were not recognised as suitable, whereas their acceptor and donor counterparts were classed as ideal. Indeed, there were other donor and acceptor sites in all three introns, which were deemed more suitable than the ones used. This could explain the many splice variants found. Introns 2 and 3, where these splice variants are found, were sequenced in both patient and control. We found no differences between patient and control. However, there was a difference between the published sequence of intron 2 in both patient and control DNA that would introduce a new donor site. The possible function and tissue-specific expression of these alternatively spliced mRNAs is not known. Sequencing 1.5 kb upstream of the putative start site incorporating the promoter and its motifs did not show any mutations in the patient.

MRS4 does not appear to be the causative adPEO gene. However, it exhibits a very unusual expression pattern in the many alternative spliced transcripts observed in several species. The significance or function of these transcripts is not known. The mechanism of how cells choose splice sites is not well understood and functional splice sites do not always match the consensus sequences well, while some that match the consensus are not recognised by the splicing apparatus [8].

Mutations in the human thymidine phosphorylase gene were shown to cause MNGIE [9] and mutations in ANT1

cause adPEO [5]. These examples show that mutations in nuclear genes, encoding gene products without direct involvement in mtDNA replication, can lead to accumulation of mtDNA deletions. Our study has narrowed the candidate region for adPEO to a 3-cM gene-rich region at 10q23.3–24.3. This will facilitate the identification of the causative gene for chromosome 10 linked adPEO.

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