

ARTICLE

New variants in the mitochondrial genomes of schizophrenic patients

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The impaired mitochondrial function hypothesis in schizophrenia is based on evidence of altered brain metabolism, morphology, biochemistry and gene expression. Mitochondria have their own genome, which is needed to synthesize some of the subunits of the respiratory chain enzymes. Mitochondrial DNA (mtDNA) is maternally inherited and we observed an excess of maternal transmission of schizophrenia in a set of parent–offspring affected pairs. We therefore hypothesized that mutations in the mtDNA may contribute to the complex genetic basis of schizophrenia. The entire mtDNA of six schizophrenic patients with an apparent maternal transmission of the disease was sequenced and compared to the reference sequence. We have identified 50 variants and among these six have not been previously reported. Three of them were missense variants: *MTCO2* 7750C>A, *MTATP6* 8857G>A and *MTND4* 12096T>A. These were maternally inherited because they were also present in the mtDNA of their respective schizophrenic mothers and none of them were found in 95 control individuals. The *MTND4* 12096T>A (Leu446His) is a heteroplasmic variant present in five of the six mother–offspring patient pairs that triggers a non-conservative substitution in the ND4 subunit of complex I. Sequence alignment of 110 ND4 peptides from all eukaryotic kingdoms shows that only hydrophobic amino acids are found in this position. Moreover, leucine was conserved or substituted by an isoleucine in all mammalian species. This indicates that the presence of histidine could affect complex I activity in patients with schizophrenia.

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Introduction

Schizophrenia (MIM#181500) is a psychiatric disorder resulting from the cumulative effects of genetic and environmental risk factors. It is clinically heterogeneous

across individuals with extensive variability in symptomatic presentation, which could be due to an aetiological heterogeneity. Heritability in schizophrenia is around 80%, so genetic risk factors are important in the development of the disorder. Genetic studies have focused on chromosomal regions with both linkage and association analysis and, although there are some positive and well-replicated studies, the nuclear genomic loci identified so far only explain a small proportion of the genetic risk of schizophrenia.¹ However, eukaryotic cells also have the mitochondrial genome, which is maternally inherited and may

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contribute to the genetic basis of schizophrenia through the mitochondrial dysfunction observed in this disorder.^{2–4} The hypothesis of mitochondrial DNA (mtDNA) involvement in the genetic susceptibility for schizophrenia is supported from several perspectives. Firstly, there is some evidence to suggest higher maternal transmission of schizophrenia.^{5–8} We also observed this situation in previous studies in 56 families with two or more individuals affected with schizophrenia. Of these 56 families, 25 presented an apparently unilinal, dominant-like inheritance of schizophrenia and in 22 of them the affected parent was the mother.^{9,10} Secondly, schizophrenia shares several features with mtDNA diseases, such as pathogenesis in the brain, adult onset and variable severity.¹¹ And thirdly, fertility among male patients with schizophrenia is severely restricted and intergenerational transfer of susceptibility genes should involve the maternal lineage.¹²

Mitochondria are cellular organelles whose principal function is to produce energy as adenosine triphosphate (ATP) via the electron transport chain (ETC) and the oxidative phosphorylation system (OXPHOS). Also, these organelles participate in the biosynthesis of other cellular components, such as pyrimidines, amino acids, phospholipids and nucleotides.¹³ The human mitochondrial genome is a circular molecule of approximately 16.5 kb maternally inherited, undergoes no recombination and is haploid. It encodes for 13 crucial subunits of the 83 OXPHOS polypeptides, the 12S and 16S rRNA genes, and the 22 tRNA genes required for mitochondrial protein synthesis. MtDNA evolves and accumulates mutations more rapidly than nuclear DNA probably because of the lack of histones, the continuous generation of reactive oxygen species and the lack of efficient repairing mechanisms. One mitochondrion has an average of 5–10 mtDNA molecules, and a cell can contain thousands of mitochondria. Homoplasmy is when all cells contain identical mtDNA sequences and heteroplasmy is when different types of mtDNA genomes (mutated and non-mutated) are present in a cell or tissue.¹⁴ Defects in the mtDNA are responsible for the development of several mitochondrial diseases with very heterogeneous clinical manifestations. Most are associated with brain disorders^{15,16} and can be divided into two groups: sporadic diseases mainly due to rearrangements in the mtDNA and maternally inherited diseases due to point mutations.¹⁷

There are few studies of mtDNA in schizophrenic patients and most of them do not strongly support the association of a particular mtDNA variant with an increased risk of schizophrenia.^{18–20} However, Marchbanks *et al*²¹ have identified a heteroplasmic mtDNA sequence variant associated with oxidative stress in schizophrenia.

The aim of this study is to investigate whether patients with an apparent maternal transmission of schizophrenia carry mtDNA mutations that could contribute to the mitochondrial dysfunction described in schizophrenia. To

test this hypothesis, we fixed three objectives: (1) to sequence the entire mitochondrial genome of patients with an apparent maternal transmission to find cytoplasmic-inherited mutations; (2) to test the presence of these mutations in a control population; and (3) to estimate, by bioinformatic methods, the possibility that these mutations could affect the structure or both the structure and function of proteins.

Subjects and methods

The study was carried out with the approval of the Hospital Ethics Committee and written informed consent was obtained from schizophrenic patients and control individuals after full explanation of the procedures. Blood samples were obtained from peripheral blood and the DNA isolated from leucocytes by the Pure Gene Kit (Gentra).

Subjects

Patients and controls were from the same geographical region and had similar ethnic background. We studied six unrelated schizophrenic patients and their schizophrenic mothers, all of them diagnosed according to ICD-9 criteria and treated at the Hospital Psiquiàtric Universitari Institut Pere Mata (Reus, Spain). Demographic and clinical data are presented in Table 1. Patients were originally selected for linkage analysis, as we have previously described.⁹ They did not have any medical condition compatible with any known mitochondrial disease. A total of 47 males and 48 females composed the control group; the age range at the time of sample collection was 20–74 (mean age, 43 years; SD, 14 years). All scored 0 in the Goldberg General Health Questionnaire (GHQ-28) and they had no life history of psychiatric symptoms or first-degree relatives with psychiatric disorders. They were collected as we have previously described.²²

MtDNA sequencing

The entire mtDNA of the six offspring patients was amplified into 24 completely overlapping PCR fragments using previously described primers and checked against Genbank to ensure specificity for the mitochondrial genome²³ (details for PCR amplification conditions are available on request from the corresponding author). We used the dye-labelled Dideoxy Terminator Cycle Sequencing kit (CEQ™ DTCS, Beckman Coulter) for sequencing reactions and the PCR products were purified by ethanol precipitation and run on a CEQ2000 DNA Analysis System (Beckman Coulter).

Variant identification

The resulting sequences were aligned and compared to the revised Cambridge Reference Sequence²⁴ using the Sequencher 3.0. Software (Gene Codes Corporation). To

Table 1 Demographic and clinical variables from patients and their mothers at the time of sample collection

| ID | Age | Gender | Schizophrenia subtype ^a | Age at onset ^b | Type of onset ^c | Course of illness ^c |
|-----------|-----|--------|------------------------------------|---------------------------|----------------------------|--|
| Mother 1 | 66 | Female | Undifferentiated | 32 | Insidious | Chronic with impairment |
| Patient 1 | 45 | Male | Undifferentiated | 23 | Insidious | Chronic (continuous) |
| Mother 2 | 64 | Female | Undifferentiated | 36 | Insidious | Chronic with impairment |
| Patient 2 | 40 | Female | Simple | 20 | Insidious | Chronic (continuous) |
| Mother 3 | 72 | Female | Residual | 38 | Insidious | Chronic (continuous) |
| Patient 3 | 44 | Female | Undifferentiated | 20 | Acute | Multiple episodes with partial remission |
| Mother 4 | 73 | Female | Paranoid | 43 | Insidious | Chronic (continuous) |
| Patient 4 | 41 | Female | Paranoid | 22 | Insidious | Multiple episodes with partial remission |
| Mother 5 | 62 | Female | Paranoid | 25 | Acute | Multiple episodes with partial remission |
| Patient 5 | 34 | Female | Undifferentiated | 13 | Insidious | Multiple episodes with partial remission |
| Mother 6 | 72 | Female | Residual | 32 | Insidious | Chronic (continuous) |
| Patient 6 | 46 | Female | Hebephrenic | 26 | Insidious | Multiple episodes with partial remission |

^aSchizophrenia subtype according to ICD-9 criteria.

^bAge at onset was defined as the age at which the first prominent psychotic symptom (congruent with ICD-9 criteria) was noted.

^cAccording to the Association for Methodology and Documentation in Psychiatry (AMDP) scale system.

determine whether the sequence variants identified in the mitochondrial genomes of schizophrenic patients had previously been described, we checked their presence in the Human Mitochondrial Genome Database (www.mitomap.org), the Human Mitochondrial Genome Polymorphism Database (www.giib.or.jp/mtsnp/index_e.shtml), the alignment of 928 European sequences (<http://www.broad.mit.edu/mpg/tagger/mito.php>) and articles cited in PubMed. When the genomic change was located in an encoding region, we used the MitoAnalyzer programme (<http://www.cstl.nist.gov/biotech/strbase/mitoanalyzer.html>) to determine whether the variant triggered an amino acid change in the polypeptide sequence. Fragments containing missense variants that had not been previously reported were also analysed in their respective mothers to ensure that they were maternally inherited. We considered as heteroplasmic variants only those that presented double peaks with the same height in the electrofluorograms.

PCR-RFLP analysis

The three missense variants identified in the mtDNA of the six schizophrenic patients that had not been previously reported were analysed by restriction endonucleases in 95 control individuals and in the six schizophrenic probands and their mothers. DNA (10 ng) were amplified by PCR using AmpliTaq Gold (Roche) according to standard protocols. Primers used were the same as for the sequencing reactions for all variants except for 7750C>A where new primers were designed to better discriminate the bands in the gels (forward 5'-TTCATGATCACGCCCTCATA-3' and reverse 5'-TAAAGGATGCGTAGGGATGG-3'). Variants 7750C>A, 8857G>A, and 12096T>A remove a digestion site for *DdeI*, *BsrBI*, and *BseRI* restriction endonucleases, respectively. PCR products were digested with these enzymes for each variant with standard protocols and the supplier's recommendations (New England Biolabs). The DNA from the schizophrenic patient carrying the

variant was used in each plate as positive controls. Digested products were electrophoresed in precasted 15% polyacrylamide gels (Novex, Invitrogene) and silver stained.

Bioinformatic analysis

To analyse to which extent the missense mutations that had not been previously reported could affect the structure or function of the proteins, we did a multiple sequence alignment of polypeptide sequences from different species to determine the residue-residue correspondences (residue position). If the residue position is conserved in all the aligned sequences from different organisms, it is assumed that it has a strong evolutive pressure against any change, and that a mutation in this conserved position could produce a dysfunction in the protein. In this case, the possible effects of this amino acid change on the protein structure were studied.

Protein sequences were obtained from SwissProt and TrEMBL protein databases (<http://www.expasy.org/sprot>). Multiple sequence alignments were constructed using the CLUSTAL W program (<http://www.ebi.ac.uk/clustalw>), version 1.74. When the sequences to be aligned were noticeably different in size, Dot-plot analysis was performed before running the multiple sequence alignment and some of them were also manually refined. Secondary structure of subunit II of cytochrome *c* oxidase (COII) was obtained from PDBsum database (www.biochem.ucl.ac.uk/bsm/pdbsum). NADH-ubiquinone oxidoreductase (complex I) has not been crystallized, the secondary structure of ND4 subunit was predicted using the following predictors available on the web: PredictProtein (<http://www.predictprotein.org>), SCRATCH (www.igb.uci.edu/tools/scratch), PSA (<http://bmerc-www.bu.edu/psa>) and PROF (<http://www.aber.ac.uk/~phiwww/prof/index.html>). The human ND4 sequence was submitted to the homology-modelling server SWISS-MODEL (<http://swissmodel>).

expasy.org) version 3.5 to obtain a prediction of the tertiary structure of this subunit.

Results

Identification of variants

Comparison of the mtDNA sequences of the six schizophrenic patients to the revised Cambridge reference sequence revealed 50 variants. Table 2 shows the non-missense variants and Table 3 shows the missense variants and their main characteristics. The assignment of these variants to the mtDNA function locations showed that they were widely distributed in the control region, RNR1 and RNR2 (12S and 16S ribosomal RNA, respectively), TT

(tRNA of threonine) and in the following subunits of the OXPHOS system: ND1, ND2, ND3, ND4, ND4L and ND5 (NADH dehydrogenase subunits 1–5 and 4L); CO1, CO2 and CO3 (cytochrome oxidase subunits 1–3); ATP6 (ATP synthase F0 subunit 6); and CYB (cytochrome *b*). We checked the presence of each variant in the Human Mitochondrial Genome Database (www.mitomap.org) and the Human Mitochondrial Genome Polymorphism Database (www.giib.or.jp/mtsnip/index_e.shtml), last check made in November 2005, and noticed that six of them had not been previously reported, one located in the 12S ribosomal RNA, and five in encoding regions. Of the five variants located in encoding regions, two were synonymous and three caused an amino-acid change. Figure 1

Table 2 Variants, except missense, present in the mtDNA sequence of the 6 schizophrenic patients studied

| Nucleotide position | Nucleotide change | Map locus | Description | Patient number |
|--|-------------------|---------------|--|----------------|
| <i>Non-previously reported nucleotide variants</i> | | | | |
| 845 | A→G | MTRNR1 | 12S ribosomal RNA | 3 |
| 3984 | C→T | MTND1 | NADH dehydrogenase subunit 1 | 3 |
| 4599 | C→T | MTND2 | NADH dehydrogenase subunit 2 | 4 |
| <i>Previously reported nucleotide variants</i> | | | | |
| 146 | T→C | MTHV2/MTOHR | Hypervariable segment 2/H-strand origin | 4 |
| 152 | T→C | | | 4 |
| 489 | T→C | MTATT/MTDLOOP | Membrane attachment site/displacement loop | 6 |
| 498 | del C | | | 4 |
| 513 | G→A | MTDLOOP | Displacement loop | 6 |
| 813 | A→G | MTRNR1 | 12S ribosomal RNA | 6 |
| 1189 | T→C | | | 4 |
| 3010 | G→A | MTRNR2 | 16S ribosomal RNA | 2 |
| 3480 | A→G | MTND1 | NADH dehydrogenase subunit 1 | 4 |
| 4793 | A→G | MTND2 | NADH dehydrogenase subunit 2 | 3 |
| 6446 | G→A | MTCO1 | Cytochrome <i>c</i> oxidase subunit I | 6 |
| 6671 | T→C | MTCO2 | Cytochrome <i>c</i> oxidase subunit II | 6 |
| 6680 | T→C | | | 6 |
| 6776 | T→C | | | 5 |
| 8119 | T→C | | | 5 |
| 8155 | G→A | | | 1 |
| 9093 | A→G | MTATP6 | ATP synthase F0 subunit 6 | 4 |
| 9540 | T→C | MTCO3 | Cytochrome <i>c</i> oxidase subunit III | 6 |
| 9698 | T→C | | | 4 |
| 10400 | C→T | MTND3 | NADH dehydrogenase subunit 3 | 6 |
| 10550 | A→G | MTND4L | NADH dehydrogenase subunit 4L | 4 |
| 11299 | T→C | MTND4 | NADH Dehydrogenase subunit 4 | 4 |
| 11377 | G→A | | | 4 |
| 12705 | C→T | MTND5 | NADH dehydrogenase subunit 5 | 6 |
| 15043 | G→A | MTCYB | Cytochrome <i>b</i> | 6 |
| 15172 | G→A | | | 6 |
| 15301 | G→A | | | 6 |
| 15930 | G→A ^a | MTTT | tRNA Threonine | 1 |
| 16129 | G→A | MTHV1/MT7SDNA | Hypervariable segment 1/7S DNA | 6 |
| 16183 | A→C | MTHV1 | Hypervariable segment 1 | 6 |
| | A→AC | | | 4 |
| 16189 | T→C | MT7SDNA | 7S DNA | 6 |
| 16223 | C→T | | | 6 |
| 16224 | T→C | | | 4 |
| 16249 | T→C | | | 6 |
| 16519 | T→C | | | 1,2,3,4,5,6 |

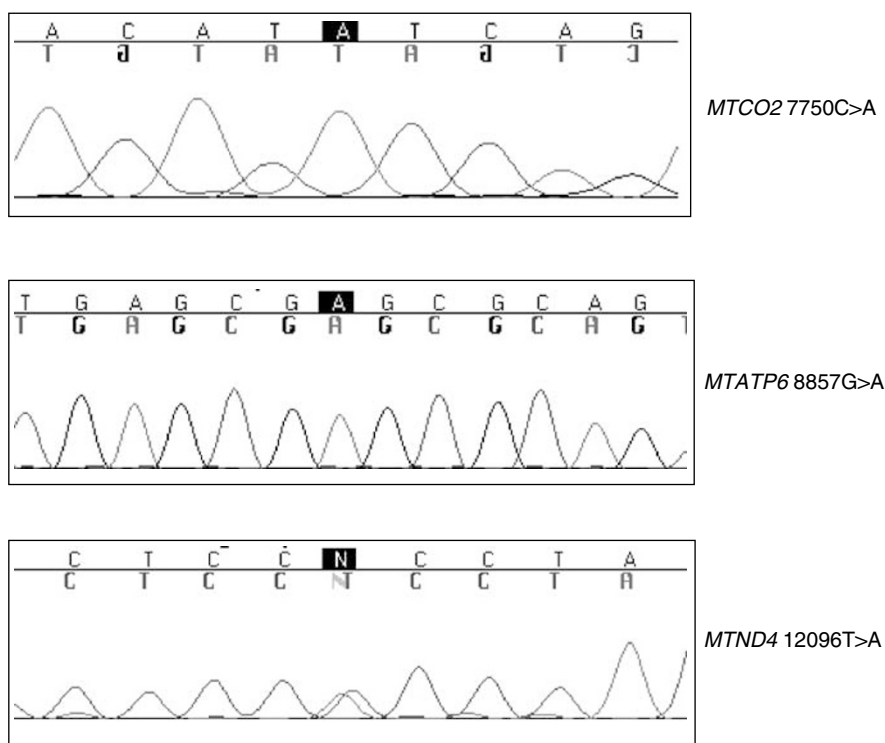
^aHeteroplasmic variant. All previously reported variants were present in the Human Mitochondrial Genome Database (www.mitomap.org) in November 2005 except the nucleotide changes at position 813 described in Kirk *et al*²⁵; 498, 9093 and 15 172 described in Finnilä *et al*³²; and 6446 and 8119 described in Hernstadt *et al*.³³

Table 3 Main characteristics of the missense variants identified in the mtDNA sequence of the six schizophrenic patients and their schizophrenic mothers

| Nucleotide position | Nucleotide change | Gene | Amino acid change | Polypeptide | Complex | Patient number | Mother number |
|--|-------------------|---------------|-------------------|--|---------|----------------|---------------|
| <i>Non-previously reported missense variants</i> | | | | | | | |
| 7750 ^a | C→A | <i>MTCOII</i> | Ile55Met | Cytochrome c oxidase subunit II (COII) | IV | 1 | 1 |
| 8857 | G→A | <i>MTATP6</i> | Gly111Ser | ATP synthase F0 subunit 6 (ATP6) | V | 4 | 4 |
| 12096 | T→A ^b | <i>MTND4</i> | Leu446His | NADH Dehydrogenase subunit 4 (ND4) | I | 1, 3, 4, 5, 6 | 1, 3, 4, 5, 6 |
| <i>Previously reported missense variants</i> | | | | | | | |
| 8701 | A→G | <i>MTATP6</i> | Thr→Ala | ATP synthase F0 subunit 6 (ATP6) | V | 6 | NA |
| 8860 | A→G | | Thr→Ala | | | 4 | NA |
| 9055 | G→A | | Ala→Thr | | | 4 | NA |
| 11016 | G→A | <i>MTND4</i> | Ser→Asn | NADH dehydrogenase subunit 4 (ND4) | I | 3 | NA |
| 10398 | A→G | <i>MTND3</i> | Thr→Ala | NADH dehydrogenase subunit 3 (ND3) | I | 4, 6 | NA |
| 12403 | C→T | <i>MTND5</i> | Leu→Phe | NADH dehydrogenase subunit 5 (ND5) | | 6 | NA |
| 12950 | A→C | | Asn→Thr | | | 6 | NA |
| 15884 | G→A | <i>MTCYB</i> | Ala→Thr | Cytochrome <i>b</i> (Cyt <i>b</i>) | III | 6 | NA |

^aThis variant is not reported as polymorphism or mutation but is present in the Swedish reference sequence (X93334) in the Human Mitochondrial Genome Database (www.mitomap.org) in November 2005.

^bHeteroplasmic mutation. All previously reported variants were present in Mitomap except the nucleotide change 12950 A>C, which was first described in patients with bipolar disorder.²⁷ NA: not analysed.

**Figure 1** Electrofluorograms of variants *MTCOII* 7750C>A, *MTATP6* 8857G>A and *MTND4* 12096T>A identified in schizophrenic patients.

shows the electrofluorograms with the nucleotide sequences coding for the non-previously reported missense variants. These missense variants were maternally inherited because they were also identified in the mtDNA of their respective mothers. Moreover, they were not located in the

pseudogenes of the nuclear genome because the primers used for the PCR reactions do not amplify the nuclear product when DNA from rho zero cells (kindly provided by Dr Attardi of Caltech Pasadena) is used (data not shown). As Tables 2 and 3 show, the number of variants in the

patients we studied is highly variable. Of the 50 variants, patients number 4 and 6 carried 19 and 24 of them, respectively, while patients number 1, 2, 3 and 5 carried five, two, six and four variants, respectively. Patients 1, 2, 3 and 5 belong to haplogroup H, patient 4 belongs to haplogroup K and patient number 6 to haplogroup W.

Two variants were observed in heteroplasmy. The *MTT* 15930 G>A was a previously reported variant affecting the tRNA of threonine. The *MTND4* 12096 T>A was a missense mutation located in the ND4 subunit of complex I and, as far as we know, it has not been reported before. However, we cannot rule out the possibility that other heteroplasmic variants were present in a proportion of <50% (according to the height of the peaks of the electrofluorograms).

Analysis of missense variants in control population

The three non-previously reported missense variants identified in the mtDNA of the schizophrenic patients were the object of further studies. These missense variants were: *MTCO2* 7750 C>A, *MTATP6* 8857 G>A and *MTND4* 12096 T>A. They were analysed in 95 control individuals by PCR-RFLP. None of these variants were found in any of the control subjects. The same PCR-RFLP analysis confirmed the presence of each variant in the same mother-offspring pair as was found by sequence analysis. Moreover, for the *MTND4* 12096 T>A heteroplasmic variant, the analysis showed undigested fragments corresponding to the presence of the variant in patients 1, 3, 4, 5 and 6 and their respective mothers (Figure 2). This variant was first identified in the electrofluorograms of patients 4 and 5. Revision of the electrofluorograms revealed two peaks in this position for patients 1, 3 and 6, although the proportion was <50%. None of the 95 control individuals presented undigested bands, which indicates that they did not have the mutation.

Bioinformatic analysis of non-previously reported missense variants

The *MTCO2* 7750C>A mutation leads to Ile55Met substitution in subunit II (COII) of cytochrome *c* oxidase

(complex IV). Although this is a conservative substitution, as the main physicochemical properties of both amino acids are the same, the multiple sequence alignment of 92 mammalian COII sequences indicated that methionine is never present, since only isoleucine or threonine is found in position 55 (Figure 3a). According to the secondary structure released in the PDBsum database, Ile55 belongs to a loop between helix alpha 1 and helix alpha 2 of COII located in the mitochondrial matrix domain. The *MTATP6* 8857G>A mutation leads to the Gly111Ser substitution in subunit 6 of the ATP synthase (complex V). Multiple sequence alignment of 96 eukaryotic ATP6 sequences showed that semiconservative substitutions are permitted in position 111 (results not shown). Furthermore, serine was found in this position in two primates (*Pongo pygmaeus abelii*, and *Pongo pygmaeus pygmaeus*). The *MTND4* 12096T>A mutation triggers Leu446His non-conservative substitution in the ND4 subunit of NADH-ubiquinone oxidoreductase (complex I). Multiple sequence alignment of 110 ND4 sequences from all eukaryotic kingdoms (Figure 3b) showed that a hydrophobic residue invariably occupies position 446, so histidine, which is a hydrophilic amino acid, is never present. Moreover, in all (30) aligned mammalian sequences Leu446 was conserved (29) or substituted by an isoleucine (1), which is a conservative substitution.

Discussion

We have studied the entire mtDNA of six schizophrenic subjects with a strict matrilineal transmission pattern of the illness to test the hypothesis that mutations present in their mitochondrial genomes contribute to the genetic basis of the illness. We found considerable sequence diversity. When we compared the mtDNA of the six schizophrenic patients with the revised Cambridge sequence, we found 50 variants. This represents one variant every 331 bp. All variants were distributed along the mitochondrial genome and did not accumulate in a specific region. Of the 50 variants, six have not been previously reported and three of them were missense mutations also

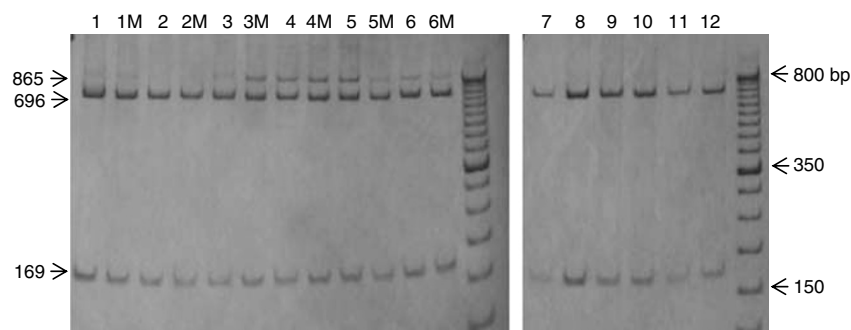


Figure 2 Silver-stained polyacrylamide gels of variant 12096 T>A analysed by PCR-RFLP in patients and their respective mothers (lines 1–6 M) and in six control individuals (lines 7–12).

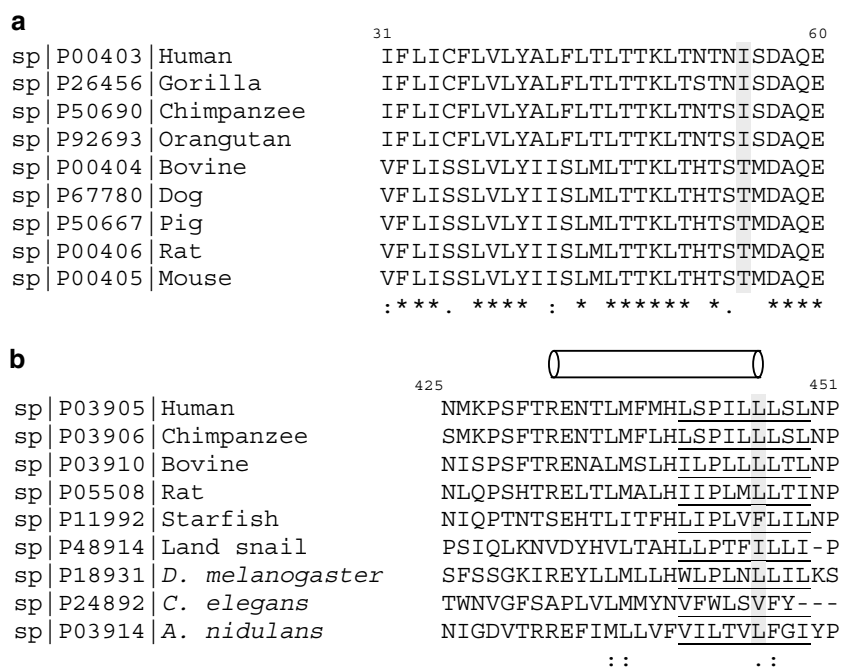


Figure 3 Multiple sequence alignment of cytochrome-c-oxidase subunit II (COII) and NADH-ubiquinone oxidoreductase subunit 4 (ND4). (a) Representative fragment of 92 COII mammalian sequences where Ile55Met occurs. Shaded column shows that position 55 is invariably occupied by isoleucine (I) or threonine (T). (b) Representative fragment of ND4 eukaryotic sequences where Leu446His occurs. Shaded column shows that only hydrophobic amino acids are found in position 446 and the conservation of leucine (L) or isoleucine (I) in mammals. The underlined residues represent the non-charged cluster around position 446. Residues 432 to 447, predicted as alpha helix, are overdrawn with a cylinder. Numbers appearing above the multiple sequence alignment correspond to the amino acid numbering of human sequence; (sp) SwissProt accession number; (*) residues in that column are identical in all sequences in the alignment; (:) residues presenting conservative substitutions; (.) residues presenting semiconservative substitutions.

present in the mtDNA of their schizophrenic mothers and none were present in the 95 control subjects studied. Variants *MTCO2* 7750C>A (Ile55Met) and *MTATP6* 8857G>A (Gly111Ser) that were each found in one mother/offspring schizophrenic pair, could be rare mutations with a frequency of <1% in the general population. This is not the case for the other missense variant identified in the ND4 subunit, the *MTND4* 12096T>A (Leu446His) heteroplasmic mutation, which was found in five of the six mother/offspring schizophrenic pairs but not in 95 control individuals. However, we should point out that none of these three variants is present in the 95 control individuals we studied. In this respect, we also found two other missense variants that had previously been described as polymorphisms in patients with bipolar disorder.²⁵ These were the *MTND5* C12403T (Leu23Phe) and *MTND5* A12950C (Asn105Thr), and they were identified in two out of 23 and 24 bipolar patients, respectively. However, they were not found in QJ; a subsequent analysis of 94 control individuals. We can, therefore, conclude that variants located in subunits of the NADH-ubiquinone oxidoreductase (complex I) could be associated to psychotic disorders. Further studies in larger samples are needed to elucidate this hypothesis. We should also point out that

patients 4 and 6, who belong to haplogroups K and W, respectively, present 82% of the missense variants and 80% of the non-missense variants we identified. Another possibility is that the presence or accumulation of several variants in the mtDNA contributes to the genetic basis of specific symptoms of schizophrenia. In support of this, in a study carried out with mice, it was found that polymorphisms present in subunits of complexes IV and I could explain the behavioural differences among the cognitive tasks they performed.²⁶ They found direct evidence of mtDNA involvement in cognitive functioning and cognitive impairment is one of the main clinical symptoms in schizophrenic patients.²⁷

The results of multiple sequence alignments suggest that *MTATP6* 8857G>A (Gly111Ser) would not disrupt the structure or activity of complex V. Position 111 was not conserved among mammals but also serine was found in this position in primate sequences. As with *MTATP6* 8857G>A, multiple sequence analysis also suggest that *MTCO2* 7750C>A (Ile55Met) would not disrupt complex IV structure or function. Anyway, position 55 of COII is invariably occupied by isoleucine or threonine in all aligned mammalian sequences (Figure 3a). In summary, bioinformatic analysis has shown that *MTCO2* 7750C>A

and *MTATP6* 8857G>A appear more likely to be non-previously reported mtDNA variants rather than mutations that directly distort the structure or function of complexes IV and V, respectively. Nevertheless, we cannot completely rule out the possibility that these variants may contribute to the vulnerability to develop schizophrenia, since we found the variants in schizophrenic patients but not in the control population. On the other hand, Leu446His in ND4 may trigger a reduction in the rate of ATP production. The multisequence alignment analysis showed that position 446 of ND4 is invariably occupied by a hydrophobic amino acid in all the 110 aligned sequences from all eukaryotic kingdoms (Figure 3b). Moreover, only leucine (29) or isoleucine (1) was found in position 446 in the 30 mammalian aligned sequences. These results indicate a strong evolutionary pressure against the presence of a polar amino acid such as histidine in this position. These results were reinforced by the analysis of the secondary structure prediction. According to this analysis, Leu446 belongs to an alpha helix (Figure 3b) that comprises residues 432 to 447 and actively participates in its stabilization through interactions with the lateral chains of other hydrophobic residues.²⁸ Since histidine is a polar amino acid, it cannot form these stabilizing hydrophobic interactions, so the predicted alpha helix structure would be compromised. The bioinformatic analysis therefore suggests that introducing a histidine in position 446 of ND4 in schizophrenic patients could distort the ND4 subunit structure. Several studies indicate that ND4 is essential for the assembly of complex I²⁹ and could be involved in the ion channel formation for H⁺ pumping within the enzyme³⁰ and the introduction of a histidine in position 446 of the ND4 subunit could affect NADH-ubiquinone oxidoreductase activity. Since complex I activity in schizophrenic patients has been related to psychotic symptomatology,³¹ we suggest that the *MTND4* 12096T>A mutation could participate in this relationship. The fact that this mutation is not found in homoplasmism also reflects the importance of the change in the micro-environment of the protein. Another heteroplasmic variant (*MTND4* 12027T>C) also located in the ND4 subunit and associated to schizophrenia and oxidative stress has recently been described,²¹ which indicates that this subunit could be important for schizophrenia.

Our results need to be validated and replicated in many more samples. It would also be interesting to analyse, in case-control studies, other variants for example, *MTND5* C12403T and *MTND5* A12950C. These variants, which were first described in bipolar patients, were not found in the control population but were identified in the schizophrenic patients we studied. And also, to determine whether the accumulation of specific mitochondrial variants is associated to schizophrenia and to the cognitive impairment present in this disorder. In summary, our results support the hypothesis that variants in the mtDNA may be responsible for the mitochondrial dysfunction

observed in schizophrenia, at least in patients with an apparent maternal transmission of the disease but more studies must be carried out.

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