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Mitochondrial Sequence Variants in Patients with Schizophrenia

Eva Lindholm[a], *Lucia Cavelier*[a]
W. Mathias Howell[a], *Inger Eriksson*[a]
Paula Jalonen[a], *Rolf Adolfsson*[d]
Douglas H.R. Blackwood[b,c]
Walter J. Muir[b,c]
Anthony J. Brookes[a,c]
Ulf Gyllensten[a], *Elena E. Jazin*[a]

[a] Department of Medical Genetics, Uppsala University, Uppsala, Sweden;
[b] University Department of Psychiatry, Royal Edinburgh Hospital, and
[c] MRC Human Genetics Unit, Western General Hospital, Edinburgh, UK;
[d] Department of Psychiatry, Umeå University, Umeå, Sweden

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Abstract
To investigate whether mitochondrial mutations underly susceptibility to schizophrenia, we sequenced the mtDNAs of two unrelated Swedish patients with schizophrenia and low cytochrome oxidase activity and two maternally related Scottish patients from a family with suspected maternal inheritance of the disease. We found five substitutions in coding regions that have not previously been described as polymorphisms. These new substitutions were studied in 81 schizophrenic patients and five control groups from Sweden and Scotland and found to differ in frequency between populations, emphasizing the importance of using large and well-defined control materials for evaluating the association of mtDNA mutations with disease. The results do not lend strong support to the association of a particular mtDNA substitution with increased risk for schizophrenia. However, the trend towards a higher frequency of substitutions in the patients deserves further attention.

Introduction

Schizophrenia is a disorder marked by hallucinations and abnormalities of thinking, mood and behaviour often accompanied by marked social withdrawal, that affects about 1% of the population [1]. A genetic predisposition to the disease is evident from the high concordance among monozygotic as compared to dizygotic twins and the familial clustering of the disease [2]. Schizophrenia has been reported to be linked to chromosomes 6 [3–5], 3 and 8 [6], 15 [7] and 22 [8, 9]. Evidence for linkage is not conclusive at any of these loci and has not been detected in some studies [10, 11], emphasizing the probable genetic heterogeneity of the disease [12].

In recent years, a large number of human diseases have been attributed to defects in the mtDNA. Most of these diseases belong to the group of neurological diseases called mitochondrial myopathies and encephalomyopathies [13]. However, there is increasing evidence that other more common disorders might involve mtDNA mutations, such as certain types of diabetes [14, 15], deafness [16–18] and late-onset Alzheimer's disease [19, 20]. Mitochondrial mutations have also been proposed in the aetiology of maternally transmitted bipolar affective disorder [21] and Parkinson's disease [22].

Several lines of evidence indicate that defects in mitochondrial energy production could be involved in the pathogenesis of schizophrenia. Metabolic changes, such

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Fax +41 61 306 12 34
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Dr. Elena Jazin
Department of Medical Genetics, Biomedical Center, Box 589
S–751 23 Uppsala (Sweden)
Tel. +46 18 174487, Fax +46 18 526849 or +46 18 510792
E-Mail elena.jazin@medgen.uu.se

as a decrease in creatine kinase levels, have been found in the brains of schizophrenic patients, suggesting that local concentrations of ATP might be altered [23]. Schizophrenia has also been associated with neuromuscular abnormalities that cannot be attributed to medication or drug abuse [24] and retinitis pigmentosa and sensorineural deafness, both possible symptoms of mitochondrial disease [25]. We have previously reported a 50% reduction in mitochondrial COX activity in the nucleus caudatus and cortex gyrus frontalis of schizophrenia patients as compared to controls [26]. A decrease in COX activity might reflect a defect in any of the COX subunits. On the other hand, any gene that affects mitochondrial targetting, transport or metabolism might affect COX activity. Therefore, a decreased COX activity might be coupled with malfunction of any other mitochondrial or nuclear genes involved in mitochondrial function.

In this report, we have searched for mtDNA substitutions that might have a deleterious effect on oxidative phosphorylation in schizophrenic patients. To this end, the entire mtDNA genome was sequenced from (1) two unrelated Swedish schizophrenic patients with extremely low COX activities [26], and (2) two Scottish patients belonging to a family that shows a possible maternal inheritance and multiple affected offspring, a pattern that might be consistent with mtDNA mutations.

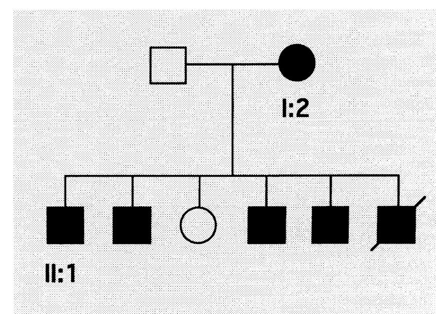

Fig. 1. Pedigree from a family with suspected maternal inheritance of schizophrenia. The mtDNA of individuals I:2 and II:1 were sequenced.

Experimental Procedures

Patient Materials

Brain samples were obtained at autopsy from a total of 12 schizophrenic individuals with a significant reduction in COX activity compared to healthy controls. These patients have been described previously [26]. Brain tissue specimens of the nucleus caudatus were dissected, freeze-dried, crushed into a coarse powder and stored at −70 °C.

Diagnoses were according to DSM-III-R (American Psychiatric Association, 1987). All schizophrenic patients had suffered from a chronic form of the disease and had been treated to varying degrees with neuroleptic drugs. The mean post-mortem delay before autopsy was 42.1 ± 18.5 h for schizophrenic patients and 90.4 ± 36.3 h for the controls.

Blood samples were obtained from individuals I:2 and II:1 in the pedigree in figure 1. These patients were diagnosed according to the criteria above. There was no history of schizophrenia in previous generations in this family. The onset of the disease was at age 35 for the mother and during their teens for generation 2. The two patients I:2 and II:3 are considered as one patient in table 3 due to the close relationship. An additional set of 68 blood samples from Swedish patients were extracted to be used for the screening of variants.

DNA Isolation

Genomic DNA was prepared from brain tissue and blood by proteinase K digestion followed by phenol extraction and ethanol precipitation. DNA amounts were estimated by DNA fluorescence (Hoechst 33258).

mtDNA Sequencing Strategy

The complete mitochondrial genome was amplified by PCR in 13 overlapping fragments (table 1) as shown in figure 2. Amplified mtDNA fragments were purified using a QIAEX Gel extraction Kit (QIAGEN) and directly sequenced using the Taq Dye Deoxy Terminator Cycle Sequencing Kit (Perkin-Elmer) employing fluorescent nucleotide terminators. A total of 120 sequence reactions was performed for each patient to determine the complete mtDNA sequence from both strands. The sequence of both strands was necessary for unambiguous sequence determination. The sequences were assembled in a contig using the program STADEN and the resulting contig was aligned to the Cambridge sequence [27].

Screening for Substitutions by Restriction Analysis and Oligo-Hybridization

The substitution at nucleotide position 2780 (C–T) was detected by digestion of PCR products with *Ava*II and the substitution at position 15758 (A–G) by digestion of PCR products with *Dde*I. The T–C change at position 3197, the A–G at position 14793, and the A–G at position 15218 were detected by hybridization with specific oligonucleotides as described [28]. The biotinylated oligonucleotides used for hybridization were:

(1) Position 3197 T–C: wild type 5′-GGTATAAT(A)CTAAGTT-G-3′ and mutant 5′-CAACTTAG(C)ATTATACC-3′. (2) Position 14793 A–G: wild-type 5′-TAACC(A)CTCATTCATCG-3′ and mutant 5′-CGATGAATGAG(C)GGTTA-3′. (3) Position 15218 A–G: wild-type 5′-ACATTGGG(A)CAGACCTA-3′ and mutant 5′-TAG-GTCTG(C)CCCAATGT-3′. Hybridization was performed with 10 pmol probe in 25 ml 2 × SSPE (0.34 *M* NaCl, 20 m*M* NaH_2PO_4, 2 m*M* EDTA pH 7.7) at 42 °C for 20 min. Washes were performed with 0.1 × SSPE/0.1% SDS at 44–52 °C, according to the T_m of the oligonucleotides. Hybridizing probe was detected using chemiluminescence (ECL, Amersham).

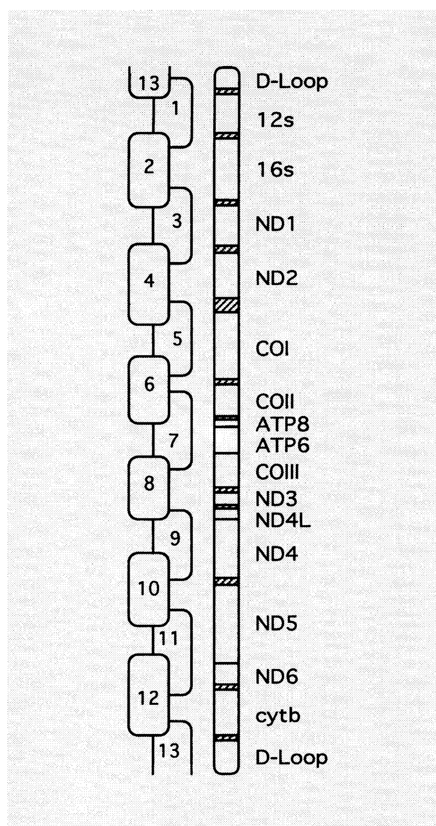

Fig. 2. Linear map of the mtDNA indicating the thirteen fragments amplified for sequencing. The sizes of the fragments and the oligonucleotides used for amplification are indicated in table 1.

Results

The mtDNA sequence was determined from brain samples of two unrelated Swedish schizophrenic patients (A and B in table 2) and blood samples of two maternally related Scottish patients (C and D in table 2, I:2 and II:1 in fig. 1). The mtDNA sequences of the patients differed from the Cambridge sequence [27] by a number of substitutions, all homoplasmic (table 2). Most of these have previously been reported as polymorphisms or errors in the Cambridge sequence [29]. Among the changes not previously reported, three of the substitutions do not alter the amino acid sequence and four are located in the displacement loop (D-loop), a region known to show the highest variability between individuals [30, 31]. For example, a six-base insertion was found at nucleotide position 524 in one of the patients, representing the largest insertion reported in the D-loop in humans. In the Swedish patients, four substitutions were found in coding regions that have not been previously described as polymorphisms; two substitutions in the 16S RNA gene (C–T at position 2780 and T–C at position 3197) and two missense mutations in the cytochrome b gene (A–G at position 14793, and A–G at position 15218). One of the two Swedish patients carried all four substitutions, while the other patient had the changes at positions 3197 and 14793. In the Scottish patients, we found a missense mutation in the cytochrome b gene (A–G at nucleotide position 15758). In addition, the Scottish individuals also

Table 1. Primers used for PCR amplification of mtDNA

Fragment	Heavy strand primer (5′ position)	Length nt	Light strand primer (5′ position)	Length nt	Fragment size, nt
1	315	20	1905	22	1590
2	1586	22	3370	22	1784
3	2897	24	4642	22	1745
4	4220	25	6156	20	1936
5	5542	30	7230	29	1688
6	6903	29	8661	20	1758
7	7743	20	9569	20	1826
8	9270	20	10748	20	1478
9	10544	23	12194	20	1650
10	11605	20	13326	20	1721
11	13002	20	14993	20	1991
12	13904	20	15777	20	1873
13	15502	20	580	20	1647

The position of the thirteen fragments relative to the mitochondrial genome is shown in figure 1. Numbering of the primer positions is according to Andersson et al. [27].

Table 2. mtDNA mutations in four schizophrenia patients

Gene	Site ntp	Base change	Change	Patient	Previously reported polymorphism
D-loop	73	A–G	–	B, C, D	yes
	199	T–C	–	C, D	yes
	207	G–A	–	C, D	yes
	263	A–G	–	A, B	yes
	316	C	Ins	A, B	yes
	460	T–C	–	C, D	no
	500	C–G		C, D	no
	524	ACACAC	Ins	A	no
16S RNA	1719	G–A	–	C, D	yes
	2780	C–T	–	A	no
	3106	C	Del	A, B, C, D	yes
	3197	T–C	–	A, B	no
ND 2	4529	A–T	Thr-Met	C, D	yes
	5054	G–A	Silent	B	no
CO II	8251	G–A	Silent	C, D	yes
CO III	9477	G–A	Val-Ile	A, B	yes
	9559	G–C	Gly-Pro	A, B	yes
	9788	C–G	Silent	B	no
ND 3	10238	T–C	Silent	C, D	yes
ND 4	11467	A–G	Silent	A, B	yes
tRNA(Leu)	12308	A–G	–	A, B	yes
ND 5	12372	G–A	Silent	A, B	yes
	13617	T–C	Silent	A, B	no
Cyt b	14793	A–G	His-Arg	A, B	no
	15218	A–G	Thr-Ala	A	yes[1]
	15758	A–G	Ile-Val	C, D	no
RNA(Thr)	15924	A–G	–	C, D	yes
D-loop	16114	C–A	–	B	yes
	16192	C–T	–	A, B	yes
	16256	C–T	–	A, B	yes
	16270	C–T	–	A, B	yes
	16286	C–T	–	A	yes
	16290	C–T	–	B	yes
	16294	C–T	–	B	yes
	16320	C–T	–	A, B	yes
	16391	G–A	–	C, D	yes
	16399	A–G	–	A, B	yes
	16519	T–C	–	C, D	yes
	16526	G–A	–	B	no

Previously reported polymorphisms were derived from the Mitochondrial Human Genome Database at Emory University in Atlanta (http://www.gen.emory.edu/mitomap.html) on March 19th, 1996 [Kogelnik et al., 1996]. We also identified in all four patients mutations at the following sites: 750 (A–G), 1438 (A–G), 2706 (A–G), 4769 (A–G), 4985 (G–A), 7028 (C–T), 8860 (A–G), 11335 (T–C), 11719 (G–A), 13702 (G–C), 14199 (G–T), 14272 (G–C), 14365 (G–C), 14368 (G–C) and 15326 (A–G). These are very frequently observed mtDNA polymorphisms and a subset of these might represent either sequencing errors or very rare mutations in the Cambridge sequence [Kogelnik et al., 1996].

ntp = Nucleotide positions as previously described [27]; A = brain sample from a woman who suffered from severe schizophrenia and died at age of 99; B = brain sample from a female patient who had severe schizophrenia and died 64 years old; C, D = blood samples from individuals I:2, and II:1 of the family shown in figure 2. About 85% of the mtDNA sequence was determined in each of these two patients. Reported sequences are L-strand base changes.

[1] Described in patients with idiopathic cardiomyopathy [35] and Leber hereditary optic neuropathy [36].

Table 3. Evolutionary conservation and frequencies of novel mtDNA changes found in the schizophrenia patients

Gene	Site ntp	Base change	Amino acid change	Patients southern Sweden n = 13[1],●	Patients northern Sweden n = 68□	Patients combined n = 81★	Controls northern Sweden n = 74	Controls middle Sweden n = 93	Controls southern Sweden n = 92	Controls combined Sweden n = 259	Controls Edinburgh n = 91	Nucleotide or amino acid conservation				
												H	B	M	X	S
16S	2780	C–T	–	0.077	0	0.013	0	0	0.022	0.008	0	C	A	C	T	C
RNA	3197	T–C	–	0.308	0.147	0.175	0.135	0.097	0.109	0.112	0.066●	T	G	T	A	#
Cyt b	14793	A–G	His-Arg	0.231	0.132	0.150	0.095	0.032●●★★,□	0.054★	0.058●★	0.012●●★★★,□□	His	Asn	His	Asn	Ser
	15218	A–G	Thr-Ala	0.154	0.088	0.100	0.081	0.022★	0.011●★,□	0.035★	0●★★,□□	Thr	Thr	Thr	Asn	Thr
	15758	A–G	Ile-Val	0.077	0.015	0.025	0.014	0.043	0.022	0.027	0.045	Ile	Ile	Ile	Ile	Ile

n = Number of individuals studied; H = human *(Homo sapiens)*; B = bovine *(Bos taurus)*; M = mouse *(Mus musculus)*; X = xenopus *(Xenopus laevis)*; S = sea urchin *(Stronylocentrotus purpuratus)*; # = no corresponding position; ntp = nucleotide positions as previously described [27].

● □ ★ = Fisher's exact test, p < 0.05 (one symbol), p < 0.01 (two symbols), p < 0.001 (three symbols) for the comparisons between each of the five control groups and the patients from southern Sweden (●), northern Sweden (□) and patients combined (★).

[1] The Scottish patient was included in this column. In the Edinburgh controls, sites 2780 and 15758 were studied in 88 individuals and sites 14793 and 15218 were studied in 85 individuals.

had one substitution in the last base of the anticodon loop of $tRNA^{thr}$ (A–G substitution at position 15924) (table 2).

In total, among the four patients, we found five changes in coding regions that have not previously been described as polymorphisms. These substitutions occurred at positions showing varying degrees of evolutionary conservation (table 3). The frequencies of these substitutions were estimated in a set of schizophrenic patients from southern Sweden, northern Sweden and Edinburgh, UK, as well as in five groups of controls, derived from three parts of Sweden and Edinburgh, UK, in order to evaluate their association with schizophrenia (table 3). The Fisher's exact test was calculated for all possible comparisons between frequencies in the patients and the control groups. Among the five substitutions, the variants at positions 14793 and 15218 showed a significantly higher frequency in the combined patients as compared with the combined controls from Sweden (n = 259) (Fisher's exact test p = 0.016 and p = 0.035, respectively). However, the patients from northern Sweden did not show any significant difference in frequency as compared to the controls from the same area (not shown). Thus, the differences between the combined patients and controls might reflect population stratification, rather than disease association. On the other hand, the substitutions at positions 3197, 14793 and 15218 showed significantly higher frequencies in the patients as compared to the controls from southern Sweden. These results emphasize the need to exert great care in choosing an appropriate control population when evaluating mtDNA mutations. For example, the substitution at 2780 was only found in southern Sweden, while 15218 was more prevalent in northern Sweden.

Discussion

We have examined the mtDNA sequences from two unrelated patients and a pair of maternally related patients with schizophrenia. This study followed earlier findings that defects in mitochondrial energy production could be associated with schizophrenia [23–26]. Our hypothesis was that mtDNA sequence variants might underlie the disease susceptibility in these patients.

Our results revealed the presence in the schizophrenic patients of five mtDNA sequence variants not previously reported as polymorphisms. Three of these are located in the cytochrome b gene. Many polymorphic positions were found in the cytochrome b gene [29] but they usually do not involve missense mutations in conserved amino acid residues [16, 22, 32]. On the other hand, three missense mutations in the cytochrome b gene have been associated with different diseases: substitution 15257 (G–A), 15812 (G–A) [29] and 15615 (G–A) [33]. In our study, the three cytochrome b substitutions were found to affect moderately conserved positions (at nucleotides 14793 and 15218) and a very conserved position (at nucleotide 15758) [34]. The 14793 change alters an amino acid in the N-terminal domain of cytochrome b, the 15218 change modifies a residue in the second intracellular loop and the 15758 change is located in the seventh transmembrane domain. The 15218 substitution has previously been de-

scribed in patients with idiopathic cardiomyopathy [35] and Leber hereditary optic neuropathy [36]. A mutation was also found in the last base of the anticodon loop of tRNA thr (position 15924). The 15924 mutation was first described in patients with fatal infantile respiratory enzyme deficiency [37], but has recently been detected in about 11% of control subjects, suggesting that it is a polymorphism [9]. The 3197 substitution, in the 16S RNA, has previously been described in a patient with ischaemic colitis. It has been suggested that this substitution may modify the expression of another mitochondrial mutation [38]. The frequencies of the five substitutions found were determined in 81 patients and five control groups. The combined patients and controls showed significant differences at two positions. However, when the groups of patients and controls from the same area were compared, significant differences were only found between patients and controls from southern Sweden. The frequency differences observed among regions within the same country stress the importance of using a large and well defined control material when assessing the association of mitochondrial sequence variants with disease. Our results indicate that a sample of 100–200 controls, a frequently used sample size in many other studies [22, 39, 40], might not be enough to evaluate the importance of mitochondrial substitutions.

When compared to the distribution of continent-specific mtDNA variants [41], the mtDNAs of the schizophrenia patients were found to resemble lineages of European origin most closely, in agreement with the Swedish and Scottish origin of the patients (data not shown). On the other hand, the Swedish and Scottish mtDNAs are not found on any of the major branches of the European mtDNA clade. Therefore, the possibility remains that the mtDNA of patients with schizophrenia might belong to the same ancestral lineage, as recently suggested for some late onset Alzheimer patients [19].

In summary, our analysis of 81 schizophrenic patients and 400 controls does not lend strong support for the association of a particular mtDNA variant with increased risk for schizophrenia. However, the trend towards a higher frequency of substitutions in the patients, as well as the presence of certain substitutions in higher frequency in the group of patients from southern Sweden, deserves further attention.

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