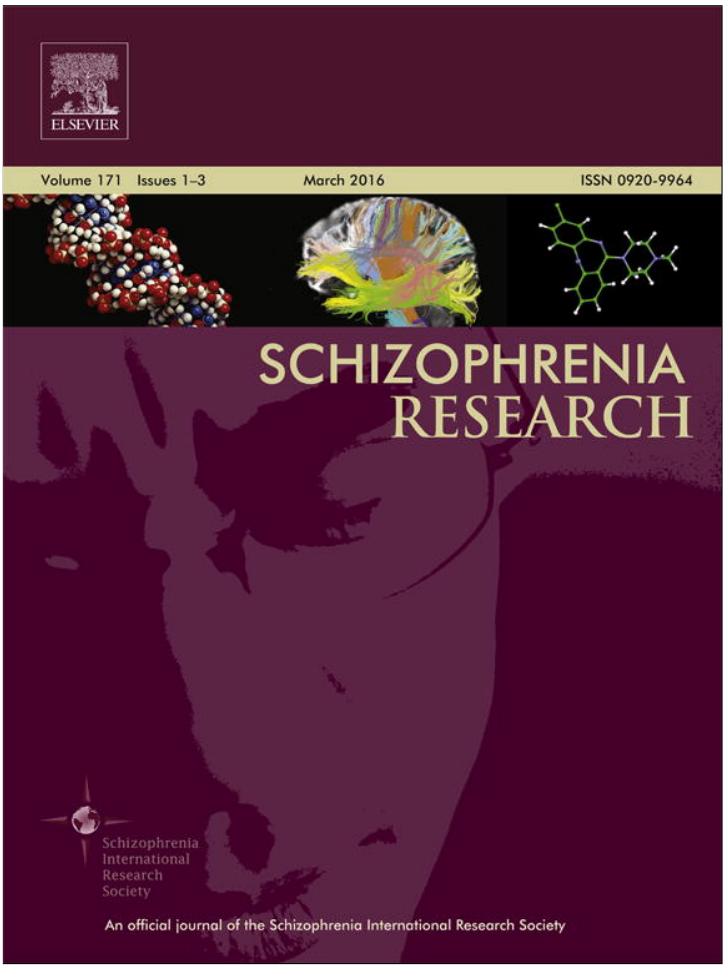

Schizophrenia Research 171 (2016) 200–206

Contents lists available at ScienceDirect

Schizophrenia Research

journal homepage: www.elsevier.com/locate/schres

Mitochondrial genome variations and functional characterization in Han Chinese families with schizophrenia

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ARTICLE INFO

Article history:
Received 4 March 2015
Received in revised form 9 December 2015
Accepted 4 January 2016
Available online 26 January 2016

Keywords:
Schizophrenia
mtDNA
Maternal inheritance
Private variant
Allotopic expression

ABSTRACT

The relationship between mitochondrial DNA (mtDNA) variants and schizophrenia has been strongly debated. To test whether mtDNA variants are involved in schizophrenia in Han Chinese patients, we sequenced the entire mitochondrial genomes of probands from 11 families with a family history and maternal inheritance pattern of schizophrenia. Besides the haplogroup-specific variants, we found 11 nonsynonymous private variants, one rRNA variant, and one tRNA variant in 5 of 11 probands. Among the nonsynonymous private variants, mutations m.15395 A>G and m.8536 A>G were predicted to be deleterious after web-based searches and *in silico* program affiliated analysis. Functional characterization further supported the potential pathogenicity of the two variants m.15395 A>G and m.8536 A>G to cause mitochondrial dysfunction at the cellular level. Our results showed that mtDNA variants were actively involved in schizophrenia in some families with maternal inheritance of this disease.

1. Introduction

Schizophrenia is a world-wide prevalent psychiatric disorder showing almost 80% heritability (Cannon et al., 1998). There are many genome-wide association studies (GWAS) aiming to investigate the risk genes for schizophrenia, and a very recent meta-analysis of the previous GWAS identified a total of 108 susceptibility loci for schizophrenia (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). However, the GWAS-identified common variants could only account for 10–30% of the variability of schizophrenia (Gunter, 2009), and the genetic risk factors for schizophrenia remain largely unknown.

The role of mitochondrial dysfunction and mitochondrial DNA (mtDNA) variants in schizophrenia has been strongly debated (Shao et al., 2008; Verge et al., 2010). Over the past decade, many studies have reported mitochondrial dysfunction in schizophrenia patients (Arvindakshan et al., 2003; Ben-Shachar and Karry, 2008; Dror et al., 2002; Karry et al., 2004; Kung and Roberts, 1999; Maurer et al., 2001; Munakata et al., 2005; Prabakaran et al., 2004; Washizuka et al., 2009). The types of dysfunction have included OXPHOS deficiency and altered expression of mitochondria related genes. This has led to the hypothesis that mitochondrial dysfunction has an important role in the causation of schizophrenia.

Since mtDNA is maternally inherited and an excess of maternal transmission of the disease has been observed in previous studies of schizophrenia (DeLisi et al., 2000; Gottesman and Bertelsen, 1989; Ichikawa et al., 2012; Verge et al., 2012), it is appropriate to conduct a comprehensive evaluation of the influence of mtDNA variants on schizophrenia. There are already several reports showing mtDNA sequence variations in patients with schizophrenia (Bamne et al., 2008; Bandelt et al., 2005; Lindholm et al., 1997; Mamdani et al., 2014; Marchbanks et al., 2003; Martorell et al., 2006; Rollins et al., 2009; Sequeira et al., 2012; Sequeira et al., 2015; Ueno et al., 2009), but no consistent results have been found and most of these studies were focused on sporadic patients rather than on maternally inherited pedigrees. Furthermore, functional characterization of the mtDNA variants in patients with schizophrenia was rarely considered, partly because the genetic code of mtDNA is different from the universal code and we lack a robust system to fulfill the aim. The allotopic expression system has been used to investigate the function of mtDNA variants in previous studies (Ellouze et al., 2008; Kaltimbacher et al., 2006), in which the mtDNA gene was converted to a nuclear-encoded version, and protein was first expressed in the cytosol and then was imported into mitochondria under the control of mitochondrial leader peptides.

Abbreviations: mtDNA, mitochondrial DNA; DSM-IV, Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition; rCRS, revised Cambridge Reference Sequence; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; ROS, reactive oxygen species.

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http://dx.doi.org/10.1016/j.schres.2016.01.011
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In order to test the effect of mtDNA variants in schizophrenia, we conducted a comprehensive mtDNA analysis of the probands of 11 families with a maternal transmission appearance of schizophrenia. We identified 11 nonsynonymous private variants, one rRNA private variant and one tRNA private variant in 5 of the 11 probands. Web-based searches and *in silico* program affiliated analysis showed that some of these private variants were very likely to be pathogenic. Functional characterization further confirmed the potential adverse functioning of these variants.

2. Materials and methods

2.1. Subjects

Subjects from eleven pedigrees (ten from Hunan Province and one from Anhui Province) were recruited in this study. Schizophrenia was regarded as being maternally inherited in the pedigree based on two criteria: (1) each family had at least three maternally related patients with schizophrenia; (2) none of the non-maternal members had schizophrenia or other psychiatric disease (Supplementary Table S1). The schizophrenia patients were diagnosed independently by two psychiatrists according to Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) and had at least a 2-year history of schizophrenia. Informed consents conforming to the tenets of the Declaration of Helsinki were obtained from each patient or their custodians prior to this study. The institutional review boards of the Kunming Institute of Zoology approved this study.

2.2. mtDNA genome sequencing and mutation analysis

Genomic DNA was extracted from peripheral blood using the standard phenol-chloroform method. The entire mtDNA sequences of the 11 probands were amplified and sequenced by using 9 pairs of overlapping primers following the same strategy and amplification and sequencing conditions in our previous study (Wang et al., 2008; Zhang et al., 2014).

mtDNA variants in each proband were recorded relative to the revised Cambridge Reference Sequence (rCRS) (Andrews et al., 1999). We followed the recently updated version of the East Asian mtDNA tree (Kong et al., 2006) and the PhyloTree (mtDNA tree Build 16; http://www.phylotree.org) (van Oven and Kayser, 2009) to classify each sample. Potential sequence abnormalities and haplogroup classification were further automatically checked by the MitoTool web server (Fan and Yao, 2013). We defined the uniqueness of each private variant by an exhaustive database search following the available guidelines (Bandelt et al., 2009). A schematic tree was reconstructed to show the relationship among these mtDNAs. Evolutionary conservation analysis for certain mtDNA variants was performed using the same approach as described in our previous studies (Ji et al., 2008; Wang et al., 2008). The TMpred program (http://www.ch.embnet.org/software/TMPRED_form.html) was used to evaluate the hydrophobicity change and the PolyPhen program (http://genetics.bwh.harvard.edu/pph/) was used to predict the potential harmfulness of each nonsynonymous private variant.

2.3. Establishment of lymphoblastoid cell line and cell culture

Blood samples from 4 subjects with mtDNA mutation m.15395 A>G (15395-1, 15395-2, 15395-3, 15395-4), and 4 subjects without any potentially deleterious mtDNA mutation (C-1, C-2, C-3, and C-4; note that the first two donors were described in our previous studies (Bi et al., 2015; Guo et al., 2012)), were collected for establishing lymphoblastoid cell lines. Lymphoblastoid cell lines were immortalized by using the Epstein–Barr virus and were cultured in RPMI 1640 (Gibco, USA, 11875) supplemented with 10% fetal bovine serum (FBS, Gibco, USA, 10099). HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA, 11966) supplemented with 10 mM galactose (Sigma-Aldrich, USA, G5388), 10% FBS (Gibco, USA, 10099), 5 mM HEPES (Sigma-Aldrich, USA, H9136) and 1 mM sodium pyruvate (Gibco, USA, 11360). All cells were cultured at 37 °C in 5% CO_2.

2.4. Allotopic expression for nonsynonymous mtDNA variants in the MT-ATP6 gene

We constructed allotopic expression vectors bearing the *MT-ATP6* variants identified in the probands to investigate the potential function of the mtDNA mutations/variants, following the optimized procedure reported in our recent study (Bi et al., 2015). In brief, the *ATP6* gene in a pCMV-Tag 4 A plasmid [kind gift from Dr. Corral-Debrinski] (Ellouze et al., 2008; Kaltimbacher et al., 2006), together with the upstream mitochondrial targeting sequence and downstream flag tag, was subcloned into FUGW vector (Addgene, England, 14883) (Lois et al., 2002). By using site-directed mutagenesis, the different mtDNA variants were introduced into the FUGW-ATP6 construct.

Each plasmid was transiently or stably transfected into HeLa cells. For transient transfection, HeLa cells cultured in 12-well plate were transfected by using 3 μL FuGENE HP transfection reagent (Roche, Switzerland, 06366236001) combined with 1 μg plasmid DNA per well, according to the manufacturer's instruction. We further constructed HeLa cells with stable expression of the *ATP6* gene using a lentivirus system following the detailed method described in our recent study (Bi et al., 2015). In brief, the lentivirus system was composed of FUGW–ATP6, packaging plasmid psPAX2 (Addgene, England, 12260) and envelope plasmid PMD2.G (Addgene, England, 12259). The successful allotopic expression and lentivirus transduction systems were verified by using the same method described in our recent study (Bi et al., 2015).

2.5. Measurement of mitochondrial function parameters

Quantitative PCR was used to determine mtDNA copy number of the lymphoblastoid cell following the approach described in our previous study (Bi et al., 2010). In brief, mtDNA content was measured by primer pair L394/H475 (5′-CACCAGCCTAACCAGATTTC-3′/5′-GGGTTGTATTGATGAGATTAGT-3′) and was normalized to the single copy nuclear β-globin gene (HBB502f/HBB614r: 5′-CTATGGGACGCTTGATGT-3′/5′-GCAATCATTCGTCTGTTT-3′). Quantitative PCR was performed on the MyiQ2 system (BioRad Laboratories, Hercules, CA, USA).

Cellular reactive oxygen species (ROS) level, ATP level, mitochondrial mass level and oxygen consumption rate were measured in HeLa cells 48 h after transfection, and in both stable expression cell strains and lymphoblastoid cell lines, by using our previously reported methods (Bi et al., 2015; Guo et al., 2012). For ROS measurement, HeLa cells and lymphoblastoid cells were respectively incubated in PBS containing 2 μM and 0.5 μM DCFH-DA probe (Sigma-Aldrich, USA, D6883) at 37 °C for 20 min, and were analyzed by using flow cytometry (BD, Vantage SE, USA) at 535 nm. For mitochondrial mass determination, cells were incubated in pre-warm medium with 100 nM MitoTracker Red FM (Molecular Probe, USA, M22425) at 37 °C for 30 min, and were analyzed by using flow cytometry (BD, Vantage SE, USA) at 644 nm. ATP level was assessed by ATP Determination Kit (Invitrogen, USA, A22066) according to the manufacturer's instruction. The results were normalized by protein concentration of each sample. Oxygen consumption rate was assessed on a Clark-type oxygen sensor (Hansatech instruments, England) at 25 °C. The cellular respiration rate was determined by recording the respiration of cells in culture medium for 10 min. Subsequently, the ATP synthase-coupled respiration rate and basal mitochondrial respiration rate were determined by sequentially adding 1 μM oligomycin (Sigma-Aldrich, USA, 75351) and 1 μM rotenone (Sigma-Aldrich, USA, R8875), each for 10 min. The final respiration rate value was normalized by the number of cells for each cell strain.

Each assay was independently performed at least three times to validate the consistency of the result. The results were normalized to the

control cells or cells transfected with FUGW vector. Statistical analysis was performed with GraphPad software (GraphPad Software, La Jolla, CA, USA) with Student's *t*-test. The data is presented as mean value with standard error of multiple independent tests.

3. Results

3.1. mtDNA genome sequence variations in Han Chinese patients with schizophrenia

In our recent studies to identify schizophrenia patients from Hunan and other provinces in China (Ma et al., 2014; Zhang et al., 2014), we were able to recruit eleven families, each of which had at least three maternally related members affected by schizophrenia. Analysis of the complete mtDNA sequences for the 11 probands from these families showed that they belonged to 10 different haplogroups, all common in East Asians (Kong et al., 2006) (Fig. 1). There was no overall statistically significant difference in the nonsynonymous/synonymous variant ratios for each mitochondrial protein-coding gene between these patients and 69 reported Chinese samples (Kong et al., 2003, 2006; Zou et al., 2010) (Supplementary Table S2). The complete mtDNA sequences of the 11 probands have been deposited in GenBank under Accession Nos. JQ411473–JQ411482 and KP668995.

We focused on the private variants which were newly accumulated in each lineage, similar to our previous study (Yu et al., 2010), to show any potential effect that might account for the disease. We excluded

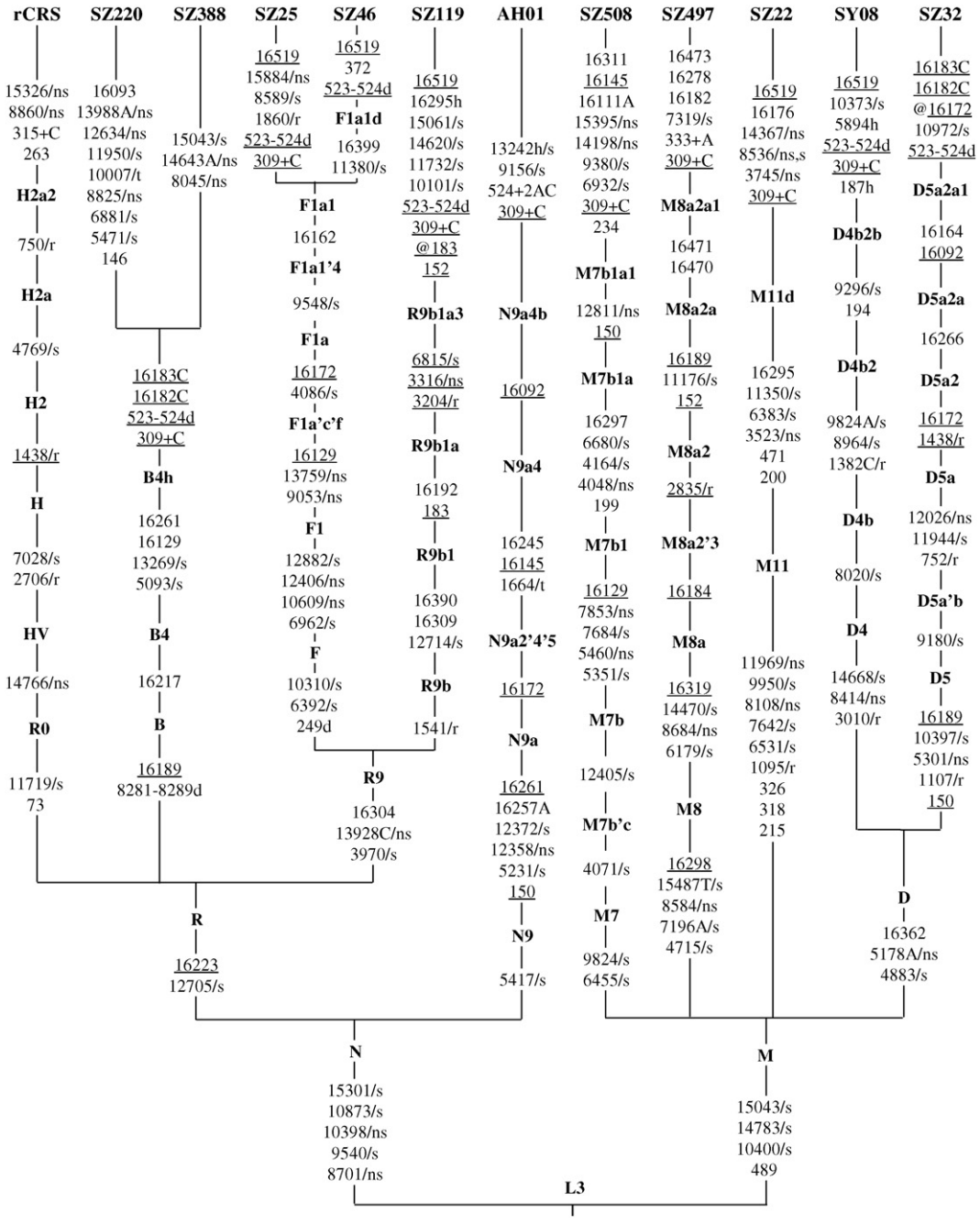

Fig. 1. Classification tree of the entire mtDNA sequences of 11 probands from families with schizophrenia, plus the revised Cambridge reference sequence (rCRS). The order of mutations/variants on each uninterrupted branch section is arbitrary. Back variants are highlighted by the prefix "@", and recurrent variants are underlined. Suffix "+C" indicates an insertion of cytosine. The heteroplasmic status was marked by "h". The synonymous and nonsynonymous mtDNA coding-region variants are denoted by "/s" and "/ns", respectively. Variations in the transfer RNA and the ribosomal RNA genes are denoted by "/t" and "/r", respectively.

private variants that were either located in the non-coding region or were synonymous from the analysis, and finally identified 13 private variants in 5 of the 11 probands, including 11 nonsynonymous variants, one tRNA variant and one rRNA variant (Table 1). Among these 13 private variants, variants m.12634 A>G, m.10007 T>C, m.15884G>A, m.14198G>A and m.3745G>A had been reported in the general populations and were haplogroup-specific variants (Table 1). These variants caused no obvious change of the transmembrane domain in hydrophobicity analysis (data not shown). In silico prediction by the PolyPhen program also showed no harmful effect for these variants. Variants m.13988 T>A, m.14643C>A, m.8045 A>G, m.1860 A>G and m.14367G>A were neither reported to be associated with disease, nor found in the general populations according to a web-based search following our previously described strategy (Bandelt et al., 2009) (Table 1). These 5 variants caused no change of hydrophobicity of the related proteins and their potential pathogenicity needs further characterization. Three variants, m.15395 A>G, m.8536 A>G and m.8825 T>C, had been reported to be associated with certain diseases (Cai et al., 2008; Chien et al., 2012; Lin et al., 2008; Ye, 2006) according to the web-based search. Evolutionary conservation analysis showed m.15395 A>G was conserved in vertebrate species, indicating that it is of potential importance. Intriguingly, m.15395 A>G was reported as a secondary mutation of LHON in Han Chinese despite that these authors did not provide any experimental data to substantiate their claim (Cai et al., 2008). PolyPhen prediction also revealed a potential damaging effect of the amino acid substitution (p.K217E) in the *MT-CYB* gene caused by m.15395 A>G. The remaining nonsynonymous variants had no damaging effect according to the PolyPhen prediction. In addition, variant m.8536 A>G was once reported as a potential risk mutation for sporadic myoclonus epilepsy in a Taiwanese (Lin et al., 2008), and m.8825 T>C was reported to be associated with type 2 diabetes (Ye, 2006). We further performed functional assay for variants m.15395 A>G, m.8536 A>G and m.8825 T>C to confirm their potential damaging effect.

3.2. Altered mitochondrial function in lymphoblastoid cell lines with mtDNA mutation m.15395 A>G

We created lymphoblastoid cell lines from proband SZ508 (15395-1) and three of her maternal relatives (15395-2, 15395-3, 15395-4) and validated the presence of mutation m.15395 A>G in these cell lines. Lymphoblastoid cell lines from 4 healthy individuals without mutation m.15395 A>G were used as controls (C-1, C-2, C-3, C-4). Mitochondrial functional parameters, including ROS level, mitochondrial mass level, mtDNA copy number, ATP level and oxygen consumption rate were determined in these lymphoblastoid cell lines. When compared against control cell lines, cells with m.15395 A>G showed a significantly decreased mitochondrial mass level ($P = 0.01$), mtDNA copynumber ($P = 0.0002$) and mitochondrial ATP synthase coupled oxygen consumption rate ($P = 0.04$) (Fig. 2A–C). Note that there were individual differences between cell lines, which might account for insignificant alterations in ATP and ROS levels between cells with and without m.15395 A>G (Fig. 2D–E).

3.3. Alteration of mitochondrial function in cells overexpressing the ATP6 gene with variants m.8536 A>G and m.8825 T>C

To assess the potential influence of the nonsynonymous private variants on mitochondrial function, allotopic expression tests were performed on two variants (m.8536 A>G and m.8825 T>C) in the *MT-ATP6* gene.

Comparing to the HeLa cells that were transfected with the wild type *ATP6* plasmid, the ATP production was consistently decreased in cells with transient allotopic expression of mutant m.8536 A>G (Fig. 3A). Mutant m.8536 A>G caused a 13% decrease in ATP production when we pooled all five independent tests together after normalization to the transfected cells with the FUGW vector ($P = 0.01$). There was no obvious change of ROS level in HeLa cells transiently over-expressing the two mutants (m.8536 A>G and m.8825 T>C) (Fig. 3B).

As variant m.8536 A>G had been reported to be associated with sporadic myoclonus epilepsy and diabetic atherogenesis (Chien et al., 2012; Lin et al., 2008) and we observed potentially functional effect of this variant in transiently transfected HeLa cells. We then constructed stable expression cell strains with m.8536 A>G to investigate further its potential function on mitochondrial activity and to eliminate potential bias caused by transient transfection. We picked 3 strains for allele m.8536 A and 2 strains for allele m.8536G. When compared with cell strains with allele m.8536 A, the cell strains with allele m.8536G exhibited a significantly increased cellular ROS level ($P = 0.03$) and decreased levels of mitochondrial mass ($P = 0.008$), cellular ATP ($P = 0.001$) and mitochondrial ATP synthase coupled oxygen consumption rate ($P = 0.047$) (Fig. 3C–F). Taken together, these results further indicated a potential deleterious role of variant m.8536 A>G on mitochondrial function.

Table 1
Private nonsynonymous, mt-tRNA and mt-rRNA variants in 11 probands from Han Chinese families with schizophrenia.

Proband[a]	Haplogroup	Nucleotide variant (amino acid change)	Gene	Reported (population context)[b]	Reported (disease context)[b]	Conservation index (CI)[c]	Haplogroup specific variant[d]
SZ220	B4h	m.13988 T>A (p.L551H)	*MT-ND5*	No	No	0.865	No
		m.12634 A>G (p.I100V)	*MT-ND5*	Yes	Yes	1	Yes (U5b2b, N11a1)
		m.10007 T>C	*MT-TG*	Yes	No	0.442	Yes (D6c1a, F1ela, H4a1a1a1a)
		m.8825 T>C (p.M100T)	*MT-ATP6*	No	Yes	1	No
SZ388	B4h	m.14643C>A (p.G11C)	*MT-ND6*	No	No	0.115	No
		m.8045 A>G (p.I154V)	*MT-CO2*	No	No	0.942	No
SZ25	F1a1	m.1860 A>G	*MT-RNR2*	No	No	0.654	No
		m.15884G>A (p.A380T)	*MT-CYB*	Yes	No	0.058	Yes (L0f2b, L0k1b, L0d2d, L5a, M1a2, M7c1b1, Z3a2, R12, R31, T1a12, B2t, H4a1a4b, H5a3, K1a4a1a, U5b1b1d, T2b32)
SZ508	M7b1a1	m.15395 A>G (p.K217E)	*MT-CYB*	No	Yes	1	No
		m.14198G>A (p.T159M)	*MT-ND6*	Yes	No	1	Yes (U5a2a1e)
SZ22	M11d	m.14367G>A (p.V103M)	*MT-ND6*	No	No	0.808	No
		m.8536 A>G (p.N4D, p.K57K)	*MT-ATP6*, *MT-ATP8*	No	Yes	0.981	No
		m.3745G>A (p.A147T)	*MT-ND1*	Yes	No	0.885	Yes (C4a3b1, H1e1c, H1bi)

[a] Complete mtDNA genomes of SZ32, SZ46, SZ497, SZ119, AH01 and SY08 contained no private nonsynonymous, mt-rRNA, and mt-tRNA variants, and were not included in the table.
[b] The search was performed on December 5, 2015 according to the described strategy (Bandelt et al., 2009)(e.g. both "T13988A mtDNA" and "13988 T>A mtDNA" were queried).
[c] The conservation index which was defined by Ruiz-Pesini et al. (Ruiz-Pesini et al., 2004), was calculated by using MitoTool (http://www.mitotool.org/) concerning 43 primate species. A CI value of 0.865 meant 86.5% of 43 primate species share the same wide type allele with human sequence (GenBank Accession number NC_012920).
[d] The column "Haplogroup-specific variant" refers to the presence or absence of the corresponding variants in the world mtDNA phylogeny displayed at http://www.phylotree.org/tree/main.htm (mtDNA tree Build 16; 19 Feb. 2014). In round brackets we indicated the haplogroup status as it defined in that tree.

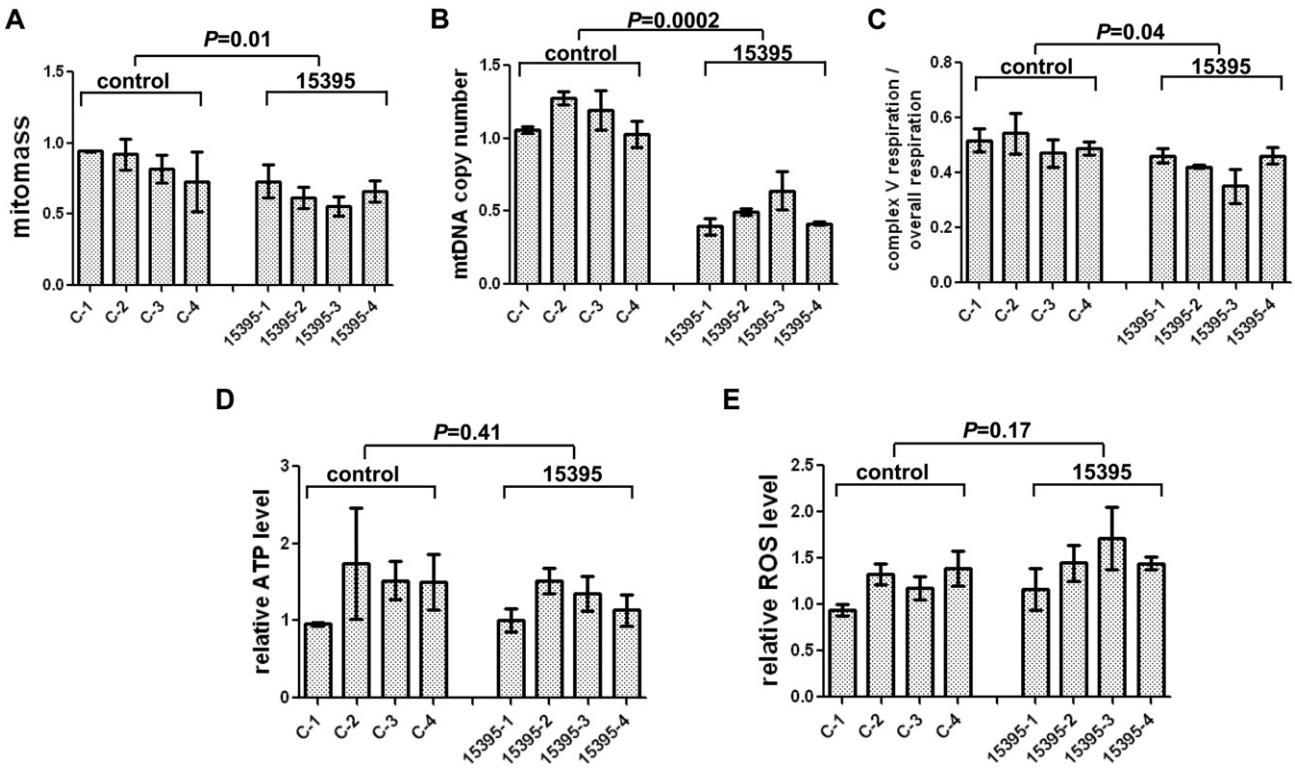

Fig. 2. Alteration of mitochondrial function in lymphoblastoid cell lines with m.15395 A>G (n = 4) and control cell lines without m.15395 A>G (n = 4). (A) Mitochondrial mass level in lymphoblastoid cell lines. Cells were incubated in 100 nM MitoTracker Red FM at 37 °C for 30 min, then were analyzed by using flow cytometry at 644 nm. (B) Determination of mtDNA copy number in lymphoblastoid cell lines. (C) Oxygen consumption rate in lymphoblastoid cell lines. About 3×10^6 cells were analyzed first in culture medium for 10 min for detection of the overall cellular respiration rate. Subsequently the ATP synthase-coupled respiration rate were determined by adding 1 μM oligomycin for 10 min. The final oxygen consumption rate was normalized by the total number of cells. (D) Cellular ATP level in lymphoblastoid cell lines. ATP level was assessed by ATP Determination Kit (Invitrogen); final ATP value was normalized by protein concentration of each cell strain. (E) Cellular ROS level in lymphoblastoid cell lines. Cells were incubated in PBS containing 0.5 μM DCFH-DA probe (Sigma-Aldrich) at 37 °C for 20 min, then were analyzed by flow cytometry at 535 nm. All results were normalized to C-1 cell line. Data was presented as mean ± standard errors of multiple independent tests. For each experiment, at least three independent tests were performed with consistent results.

4. Discussion

Schizophrenia is a major psychosis showing almost 80% heritability (Gottesman and Bertelsen, 1989). Genetic association studies based on common polymorphisms, even using a high throughput genotyping platform, only account for a little of the heritability (Gunter, 2009) and the missing heritability of schizophrenia has not been well explained (Crow, 2011; Girard et al., 2011; Ma et al., 2013). mtDNA may be a good target for consideration because the brain is the biggest energy consumer of human body (using approximately 20% of the total energy produced) and the mitochondrion is the organelle responsible for cell energy supply. Functional mutations in the mtDNA are presumed to influence cell energy metabolism, but recent studies of the mitochondrial genome in schizophrenia and bipolar disorder patients have not identified many such mutations, nor provided experimental evidence to confirm the pathogenicity (Bertolin et al., 2011).

In this study, we performed a comprehensive analysis of the mtDNA complete sequence variations in the probands from 11 Han Chinese families with a maternally inherited pattern of schizophrenia. We used the phylogenetic method to show the private nonsynonymous variants in each proband, and identified 8 rare mtDNA variants which were predicted to have the potential of adversely affecting mitochondrial function. Among these variants, m.13988 T>A, m.14643C>A, m.8045 A>G, m.1860 A>G and m.14367G>A were first reported in this study (Table 1), further functional studies are necessary to characterize their pathogenicity. Variants m.15395 A>G, m.8536 A>G and m.8825 T>C had been reported in certain diseases (Cai et al., 2008; Chien et al., 2012; Lin et al., 2008; Ye, 2006).The low frequency and high conservation index of these three variants also supported their potential pathogenicity. Furthermore, mutations m.15395 A>G and m.8536 A>G were predicted to be deleterious after web-based searches and *in silico* program affiliated analysis. Through functional characterization, we were able to show that variants m.15395 A>G and m.8536 A>G were associated with significantly decreased mitochondrial function: (1) Lymphoblastoid cell lines with m.15395 A>G exhibited significantly reduced mitochondrial mass, mtDNA copy number and ATP synthase coupled oxygen consumption rate; (2) HeLa cells with transient overexpression of m.8536 A>G showed a consistently decreased ATP level; (3) HeLa cell strains with stable expression of m.8536 A>G exhibited a significantly increased ROS level and significantly decreased mitochondrial mass level, ATP level and oxygen consumption rate. All these lines of evidence indicated that mtDNA mutations m.15395 A>G and m.8536 A>G might confer a risk to schizophrenia pathogenesis by adversely affecting mitochondrial function.

There are several limitations in this study. First, we only analyzed samples from 11 families, and the sample size was therefore relatively small. Second, we were unable to collect more clinical information for the probands and family members with the potentially pathogenic mutations, which limited our ability to understand more about the genetic and clinical correlations between certain mtDNA mutations and schizophrenia. Third, we tested the functional effect of only three variants as a way to demonstrate the efficiency of the approach, the other seven nonsynonymous variants awaited further functional characterization.

In short, we determined the mtDNA sequence variations in 11 families with maternally inherited schizophrenia and identified a total of 8 potentially functional mtDNA variants. We provided experimental data to show that variants m.15395 A>G and m.8536 A>G adversely affected mitochondrial function at the cellular level. It is worthwhile to explore further the effect of these variants on the nervous system and to define the exact mechanism of mtDNA mutations in the pathogenesis of

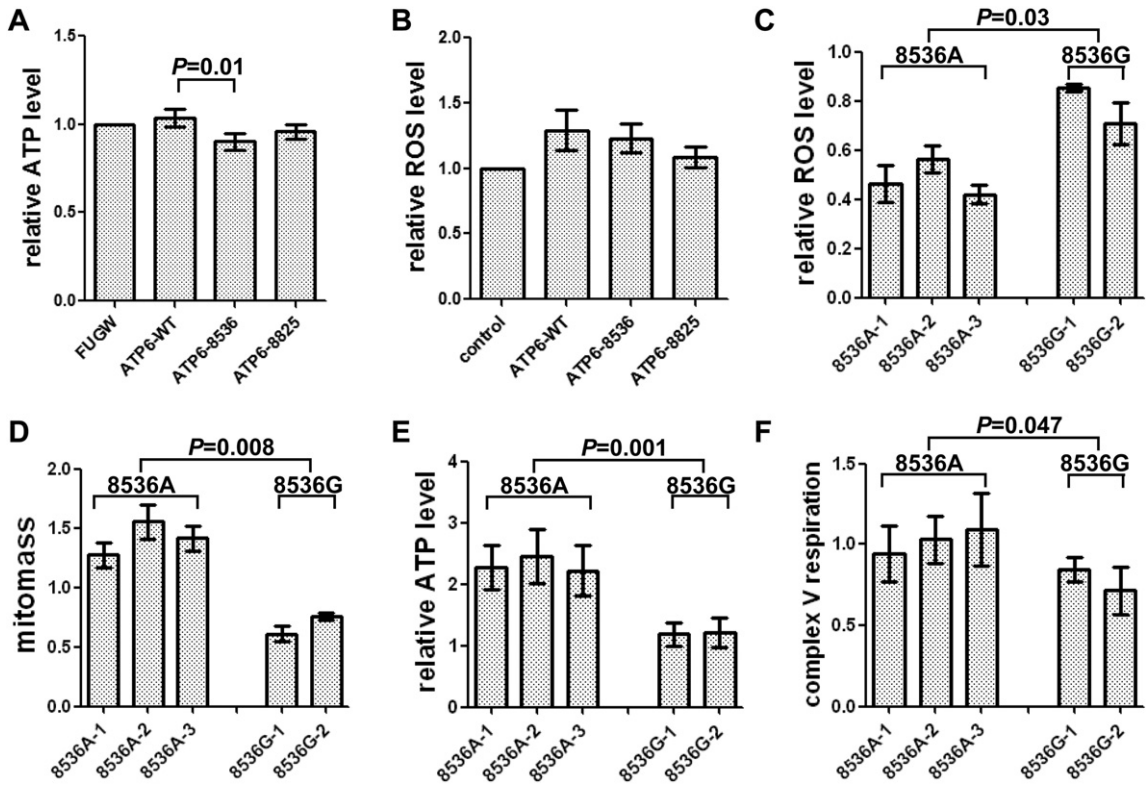

Fig. 3. Alteration of mitochondrial function in HeLa cells with allotopic expression of the *ATP6* gene. Mitochondrial function was analyzed in HeLa cells with transient (A–B) or stable (C–F) expression of the *ATP6* gene. Constructs with *MT-ATP6* gene variants (m.8536 A>G and m.8825 T>C) were transfected into HeLa cells. The ATP (A) and ROS (B) levels were measured 48 h after transfection. For HeLa cells with stable expression of the *ATP6* gene, three strains were picked for 8536 A allele and two strains were picked for 8536G allele. There was a significant increase of cellular ROS (C), decrease of mitochondrial mass (D), cellular ATP (E) and oxygen consumption rate (F) in HeLa cells with m.8536G allele compared to cells with m.8536 A allele. We followed the same condition for measurement as described in the legend of Fig. 2. Results were normalized to cells transfected with FUGW vector (A) or control cells (B–F). Data was presented as mean ± standard errors of at least three independent tests.

schizophrenia. Our current strategy of using allotopic expression provides a good paradigm to characterize the role of mtDNA mutation in schizophrenia. Along with previous reports, our results gave direct support for the active involvement of mtDNA variation in schizophrenia and related diseases.

Role of the funding source

The work was supported by the National Natural Science Foundation of China (31171225 and 31000557) and the Strategic Priority Research Program (B) of the Chinese Academy of Sciences (XDB02020003).

Contributors

Rui Bi, Wen Zhang and Shi-Yi Chen carried out the molecular genetic studies, participated in the sequence alignment and performed the statistical analysis. Rui Bi, Dandan Yu and Xiao Li carried out the functional assays. Jinsong Tang and Xiaogang Chen collected the samples. Rui Bi, Wen Zhang, Yong-Gang Yao and Xiaogang Chen drafted the manuscript. Yong-Gang Yao and Xiaogang Chen participated in the design of the study. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgments

We are grateful for the participants in this study. We thank Dr. Corral-Debrinski for their kind donation of plasmid pCMV-Tag 4A with the ATP6 gene and Mr. Ian Logan for language editing. We thank Dr. Ling Xu, Dr. Yu Fan, Mr. Jia-Qi Feng, Miss Hui-Zhen Wang, Miss Qiu-Xiang Hu and Dr. Hao Guo for technical assistance. The work was supported by the National Natural Science Foundation of China (31171225 and 31000557) and the Strategic Priority Research Program (B) of the Chinese Academy of Sciences (XDB02020003).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.schres.2016.01.011.

References

Andrews, R.M., Kubacka, I., Chinnery, P.F., Lightowlers, R.N., Turnbull, D.M., Howell, N., 1999. Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. Nat. Genet. 23 (2), 147.

Arvindakshan, M., Ghate, M., Ranjekar, P.K., Evans, D.R., Mahadik, S.P., 2003. Supplementation with a combination of omega-3 fatty acids and antioxidants (vitamins E and C) improves the outcome of schizophrenia. Schizophr. Res. 62 (3), 195–204.

Bamne, M.N., Talkowski, M.E., Moraes, C.T., Manuck, S.B., Ferrell, R.E., Chowdari, K.V., Nimgaonkar, V.L., 2008. Systematic association studies of mitochondrial DNA variations in schizophrenia: focus on the ND5 gene. Schizophr. Bull. 34 (3), 458–465.

Bandelt, H.J., Salas, A., Taylor, R.W., Yao, Y.G., 2009. Exaggerated status of "novel" and "pathogenic" mtDNA sequence variants due to inadequate database searches. Hum. Mutat. 30 (2), 191–196.

Bandelt, H.-J., Yao, Y.G., Kivisild, T., 2005. Mitochondrial genes and schizophrenia. Schizophr. Res. 72 (2–3), 267–269.

Ben-Shachar, D., Karry, R., 2008. Neuroanatomical pattern of mitochondrial complex I pathology varies between schizophrenia, bipolar disorder and major depression. PLoS One 3 (11), e3676.

Bertolin, C., Magri, C., Barlati, S., Vettori, A., Perini, G.I., Peruzzi, P., Mostacciuolo, M.L., Vazza, G., 2011. Analysis of complete mitochondrial genomes of patients with schizophrenia and bipolar disorder. J. Hum. Genet. 56 (12), 869–872.

Bi, R., Zhang, W., Yu, D., Li, X., Wang, H.Z., Hu, Q.X., Zhang, C., Lu, W., Ni, J., Fang, Y., Li, T., Yao, Y.G., 2015. Mitochondrial DNA haplogroup B5 confers genetic susceptibility to Alzheimer's disease in Han Chinese. Neurobiol. Aging 36 (3), 1604 e7.

Bi, R., Zhang, A.M., Zhang, W., Kong, Q.P., Wu, B.L., Yang, X.H., Wang, D., Zou, Y., Zhang, Y.P., Yao, Y.G., 2010. The acquisition of an inheritable 50-bp deletion in the human mtDNA control region does not affect the mtDNA copy number in peripheral blood cells. Hum. Mutat. 31 (5), 538–543.

Cai, W., Fu, Q., Zhou, X., Qu, J., Tong, Y., Guan, M.X., 2008. Mitochondrial variants may influence the phenotypic manifestation of Leber's hereditary optic neuropathy-associated ND4 G11778A mutation. J. Genet. Genomics 35 (11), 649–655.

Cannon, T.D., Kaprio, J., Lönnqvist, J., Huttunen, M., Koskenvuo, M., 1998. The genetic epidemiology of schizophrenia in a Finnish twin cohort. A population-based modeling study. Arch. Gen. Psychiatry 55 (1), 67–74.

Chien, M.C., Huang, W.T., Wang, P.W., Liou, C.W., Lin, T.K., Hsieh, C.J., Weng, S.W., 2012. Role of mitochondrial DNA variants and copy number in diabetic atherogenesis. Genet. Mol. Res. 11 (3), 3339–3348.

Crow, T.J., 2011. 'The missing genes: what happened to the heritability of psychiatric disorders? Mol. Psychiatry 16 (4), 362–364.

DeLisi, L.E., Razi, K., Stewart, J., Relja, M., Shields, G., Smith, A.B., Wellman, N., Larach, V.W., Loftus, J., Vita, A., Comazzi, M., Crow, T.J., 2000. No evidence for a parent-of-origin effect detected in the pattern of inheritance of schizophrenia. Biol. Psychiatry 48 (7), 706–709.

Dror, N., Klein, E., Karry, R., Sheinkman, A., Kirsh, Z., Mazor, M., Tzukerman, M., Ben-Shachar, D., 2002. State-dependent alterations in mitochondrial complex I activity in platelets: a potential peripheral marker for schizophrenia. Mol. Psychiatry 7 (9), 995–1001.

Ellouze, S., Augustin, S., Bouaita, A., Bonnet, C., Simonutti, M., Forster, V., Picaud, S., Sahel, J.A., Corral-Debrinski, M., 2008. Optimized allotopic expression of the human mitochondrial ND4 prevents blindness in a rat model of mitochondrial dysfunction. Am. J. Hum. Genet. 83 (3), 373–387.

Fan, L., Yao, Y.G., 2013. An update to MitoTool: using a new scoring system for faster mtDNA haplogroup determination. Mitochondrion 13 (4), 360–363.

Girard, S.L., Xiong, L., Dion, P.A., Rouleau, G.A., 2011. Where are the missing pieces of the schizophrenia genetics puzzle? Curr. Opin. Genet. Dev. 21 (3), 310–316.

Gottesman, I.I., Bertelsen, A., 1989. Confirming unexpressed genotypes for schizophrenia. Risks in the offspring of Fischer's Danish identical and fraternal discordant twins. Arch. Gen. Psychiatry 46 (10), 867–872.

Gunter, C., 2009. Complex disease: schizophrenia: missing heritability found? Nat. Rev. Genet. 10, 509.

Guo, H., Zhuang, X.Y., Zhang, A.M., Zhang, W., Yuan, Y., Guo, L., Yu, D., Liu, J., Yang, D.K., Yao, Y.G., 2012. Presence of mutation m.14484 T>C in a Chinese family with maternally inherited essential hypertension but no expression of LHON. Biochim. Biophys. Acta 1822 (10), 1535–1543.

Ichikawa, T., Arai, M., Miyashita, M., Arai, M., Obata, N., Nohara, I., Oshima, K., Niizato, K., Okazaki, Y., Doi, N., Itokawa, M., 2012. Schizophrenia: maternal inheritance and heteroplasmy of mtDNA mutations. Mol. Genet. Metab. 105 (1), 103–109.

Ji, Y., Zhang, A.M., Jia, X., Zhang, Y.P., Xiao, X., Li, S., Guo, X., Bandelt, H.J., Zhang, Q., Yao, Y.G., 2008. Mitochondrial DNA haplogroups M7b1'2 and M8a affect clinical expression of leber hereditary optic neuropathy in Chinese families with the m.11778G>A mutation. Am. J. Hum. Genet. 83 (6), 760–768.

Kaltimbacher, V., Bonnet, C., Lecoeuvre, G., Forster, V., Sahel, J.A., Corral-Debrinski, M., 2006. mRNA localization to the mitochondrial surface allows the efficient translocation inside the organelle of a nuclear recoded ATP6 protein. RNA 12 (7), 1408–1417.

Karry, R., Klein, E., Ben Shachar, D., 2004. Mitochondrial complex I subunits expression is altered in schizophrenia: a postmortem study. Biol. Psychiatry 55 (7), 676–684.

Kong, Q.P., Bandelt, H.J., Sun, C., Yao, Y.G., Salas, A., Achilli, A., Wang, C.Y., Zhong, L., Zhu, C.L., Wu, S.F., Torroni, A., Zhang, Y.P., 2006. Updating the east Asian mtDNA phylogeny: a prerequisite for the identification of pathogenic mutations. Hum. Mol. Genet. 15 (13), 2076–2086.

Kong, Q.-P., Yao, Y.-G., Sun, C., Bandelt, H.-J., Zhu, C.-L., Zhang, Y.-P., 2003. Phylogeny of east Asian mitochondrial DNA lineages inferred from complete sequences. Am. J. Hum. Genet. 73 (3), 671–676.

Kung, L., Roberts, R.C., 1999. Mitochondrial pathology in human schizophrenic striatum: a postmortem ultrastructural study. Synapse 31 (1), 67–75.

Lin, H.-M., Lu, Y.-N., Chen, W.-L., Lin, T.-T., Tsai, W.-J., Liu, C.-S., 2008. Mitochondrial DNA mutation in maternal or sporadic myoclonus epilepsy. Mitochondrion 9 (1), 73.

Lindholm, E., Cavelier, L., Howell, W.M., Eriksson, I., Jalonen, P., Adolfsson, R., Blackwood, D.H., Muir, W.J., Brookes, A.J., Gyllensten, U., Jazin, E.E., 1997. Mitochondrial sequence variants in patients with schizophrenia. Eur. J. Hum. Genet. 5 (6), 406–412.

Lois, C., Hong, E.J., Pease, S., Brown, E.J., Baltimore, D., 2002. Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. Science 295 (5556), 868–872.

Ma, L., Tang, J., Wang, D., Zhang, W., Liu, W., Liu, X.H., Gong, W., Yao, Y.G., Chen, X., 2013. Evaluating risk loci for schizophrenia distilled from genome-wide association studies in Han Chinese from central China. Mol. Psychiatry 18 (6), 638–639.

Ma, L., Wu, D.D., Ma, S.L., Tan, L., Chen, X., Tang, N.L., Yao, Y.G., 2014. Molecular evolution in the CREB1 signal pathway and a rare haplotype in CREB1 with genetic predisposition to schizophrenia. J. Psychiatr. Res. 57, 84–89.

Mamdani, F., Rollins, B., Morgan, L., Sequeira, P.A., Vawter, M.P., 2014. The somatic common deletion in mitochondrial DNA is decreased in schizophrenia. Schizophr. Res. 159 (2–3), 370–375.

Marchbanks, R.M., Ryan, M., Day, I.N., Owen, M., McGuffin, P., Whatley, S.A., 2003. A mitochondrial DNA sequence variant associated with schizophrenia and oxidative stress. Schizophr. Res. 65 (1), 33–38.

Martorell, L., Segués, T., Folch, G., Valero, J., Joven, J., Labad, A., Vilella, E., 2006. New variants in the mitochondrial genomes of schizophrenic patients. Eur. J. Hum. Genet. 14 (5), 520–528.

Maurer, I., Zierz, S., Möller, H.-J., 2001. Evidence for a mitochondrial oxidative phosphorylation defect in brains from patients with schizophrenia. Schizophr. Res. 48 (1), 125–136.

Munakata, K., Iwamoto, K., Bundo, M., Kato, T., 2005. Mitochondrial DNA 3243 A>G mutation and increased expression of LARS2 gene in the brains of patients with bipolar disorder and schizophrenia. Biol. Psychiatry 57 (5), 525–532.

Prabakaran, S., Swatton, J.E., Ryan, M.M., Huffaker, S.J., Huang, J.T.-J., Griffin, J.L., Wayland, M., Freeman, T., Dudbridge, F., Lilley, K.S., Karp, N.A., Hester, S., Tkachev, D., Mimmack, M.L., Yolken, R.H., Webster, M.J., Torrey, E.F., Bahn, S., 2004. Mitochondrial dysfunction in schizophrenia: evidence for compromised brain metabolism and oxidative stress. Mol. Psychiatry 9 (7), 684–697 643.

Rollins, B., Martin, M.V., Sequeira, P.A., Moon, E.A., Morgan, L.Z., Watson, S.J., Schatzberg, A., Akil, H., Myers, R.M., Jones, E.G., Wallace, D.C., Bunney, W.E., Vawter, M.P., 2009. Mitochondrial variants in schizophrenia, bipolar disorder, and major depressive disorder. PLoS One 4 (3), e4913.

Ruiz-Pesini, E., Mishmar, D., Brandon, M., Procaccio, V., Wallace, D.C., 2004. Effects of purifying and adaptive selection on regional variation in human mtDNA. Science 303 (5655), 223–226.

Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014. Biological insights from 108 schizophrenia-associated genetic loci. Nature 511 (7510), 421–427.

Sequeira, A., Martin, M.V., Rollins, B., Moon, E.A., Bunney, W.E., Macciardi, F., Lupoli, S., Smith, E.N., Kelsoe, J., Magnan, C.N., van Oven, M., Baldi, P., Wallace, D.C., Vawter, M.P., 2012. Mitochondrial mutations and polymorphisms in psychiatric disorders. Front. Genet. 3, 103.

Sequeira, A., Rollins, B., Magnan, C., van Oven, M., Baldi, P., Myers, R.M., Barchas, J.D., Schatzberg, A.F., Watson, S.J., Akil, H., Bunney, W.E., Vawter, M.P., 2015. Mitochondrial mutations in subjects with psychiatric disorders. PLoS One 10 (5), e0127280.

Shao, L., Martin, M.V., Watson, S.J., Schatzberg, A., Akil, H., Myers, R.M., Jones, E.G., Bunney, W.E., Vawter, M.P., 2008. Mitochondrial involvement in psychiatric disorders. Ann. Med. 40 (4), 281–295.

Ueno, H., Nishigaki, Y., Kong, Q.P., Fuku, N., Kojima, S., Iwata, N., Ozaki, N., Tanaka, M., 2009. Analysis of mitochondrial DNA variants in Japanese patients with schizophrenia. Mitochondrion 9 (6), 385–393.

van Oven, M., Kayser, M., 2009. Updated comprehensive phylogenetic tree of global human mitochondrial DNA variation. Hum. Mutat. 30 (2), E386–E394.

Verge, B., Alonso, Y., Miralles, C., Valero, J., Vilella, E., Boles, R.G., Martorell, L., 2012. New evidence for the involvement of mitochondrial inheritance in schizophrenia: results from a cross-sectional study evaluating the risk of illness in relatives of schizophrenia patients. J. Clin. Psychiatry 73 (5), 684–690.

Verge, B., Alonso, Y., Valero, J., Miralles, C., Vilella, E., Martorell, L., 2010. Mitochondrial DNA (mtDNA) and schizophrenia. Eur. Psychiatry 26 (1), 45–56.

Wang, H.W., Jia, X., Ji, Y., Kong, Q.P., Zhang, Q., Yao, Y.G., Zhang, Y.P., 2008. Strikingly different penetrance of LHON in two Chinese families with primary mutation G11778A is independent of mtDNA haplogroup background and secondary mutation G13708A. Mutat. Res. 643 (1–2), 48–53.

Washizuka, S., Iwamoto, K., Kakiuchi, C., Bundo, M., Kato, T., 2009. Expression of mitochondrial complex I subunit gene NDUFV2 in the lymphoblastoid cells derived from patients with bipolar disorder and schizophrenia. Neurosci. Res. 63 (3), 199–204.

Ye, W., 2006. Correlation Between Type 2 Diabetes Mellitus and the Variations of Mitochondrial Gene MTATP6 and MTATP8. Wenzhou Medical University, Wenzhou, p. 72.

Yu, D., Jia, X., Zhang, A.M., Guo, X., Zhang, Y.P., Zhang, Q., Yao, Y.G., 2010. Molecular characterization of six Chinese families with m.3460G>A and Leber hereditary optic neuropathy. Neurogenetics 11 (3), 349–356.

Zhang, W., Tang, J., Zhang, A.M., Peng, M.S., Xie, H.B., Tan, L., Xu, L., Zhang, Y.P., Chen, X., Yao, Y.G., 2014. A matrilineal genetic legacy from the last glacial maximum confers susceptibility to schizophrenia in Han Chinese. J. Genet. Genomics 41 (7), 397–407.

Zou, Y., Jia, X., Zhang, A.M., Wang, W.Z., Li, S., Guo, X., Kong, Q.P., Zhang, Q., Yao, Y.G., 2010. The MT-ND1 and MT-ND5 genes are mutational hotspots for Chinese families with clinical features of LHON but lacking the three primary mutations. Biochem. Biophys. Res. Commun. 399 (2), 179–185.

Supplementary data

Supplementary Table S1. Information of probands in Han Chinese families with schizophrenia

Proband	Haplogroup	Affected family members [a]
SZ22	M11d	mother and elder-brother
SZ25	F1a1	mother and elder-brother
SZ32	D5a2a1	mother and elder-sister
SZ46	F1a1d	mother and younger-brother
SZ119	R9b1a3	mother and uncle
SZ220	B4h	grandma, uncle and aunt
SZ388	B4h	mother and aunt
SZ497	M8a2a1	uncle and younger-sister
SZ508	M7b1a1	grandma and two aunts
SY08	D4b2b	mother and uncle
AH01	N9a4b	mother and grandma

[a] The family members listed here all shared the same matrilineal background as the probands, respectively. The relationship described here is relative to each proband. All of these members had been recorded and diagnosed independently by two psychiatrists according to Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) and had at least a 2-year history of schizophrenia.

Supplementary Table S2. Comparison of the nonsynonymous (NS) and synonymous (S) substitutions at the terminal branch level in the phylogenetic tree between the eleven matrilines and 69 reported complete Han Chinese mtDNAs

Gene	General population (n=69)[a]		Patients (n=11)		*P* [d]
	NS [b]	S [c]	NS [b]	S [c]	
MT-ND1	8	11	1	0	0.450
MT-ND2	4	6	0	1	1.000
MT-CO1	7	7	0	3	0.228
MT-CO2	2	5	1	0	0.375
MT-ATP8	1	4	1	0	0.333
MT-ATP6	11	2	1	3	0.053
MT-CO3	6	3	0	1	0.400
MT-ND3	1	1	0	1	1.000
MT-ND4L	0	6	0	1	1.000
MT-ND4	1	14	0	3	1.000
MT-ND5	7	19	2	1	0.220
MT-ND6	0	8	3	1	0.234
MT-CYB	5	9	2	2	1.000
Total	53	95	11	17	0.831

[a] Data were taken from our previous studies (Kong et al., 2003; Kong et al., 2006; Zou et al., 2010).

[b] NS refers to the number of non-synonymous substitutions.

[c] S refers to the number of synonymous substitutions.

[d] *P* values were determined by the Fisher's exact test with Bonferroni correction.

References

Kong, Q.-P., Yao, Y.-G., Sun, C., Bandelt, H.-J., Zhu, C.-L., Zhang, Y.-P., 2003. Phylogeny of east Asian mitochondrial DNA lineages inferred from complete sequences. Am J Hum Genet 73(3), 671-676.

Kong, Q.P., Bandelt, H.J., Sun, C., Yao, Y.G., Salas, A., Achilli, A., Wang, C.Y., Zhong, L., Zhu, C.L., Wu, S.F., Torroni, A., Zhang, Y.P., 2006. Updating the East Asian mtDNA phylogeny: a prerequisite for the identification of pathogenic mutations. Hum Mol Genet 15(13), 2076-2086.

Zou, Y., Jia, X., Zhang, A.M., Wang, W.Z., Li, S., Guo, X., Kong, Q.P., Zhang, Q., Yao, Y.G., 2010. The MT-ND1 and MT-ND5 genes are mutational hotspots for Chinese families with clinical features of LHON but lacking the three primary mutations. Biochem Biophys Res Commun 399(2), 179-185.