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ARTICLE

Analyses of the mitochondrial mutations in the Chinese patients with sporadic Creutzfeldt–Jakob disease

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Pathogenic mitochondrial DNA (mtDNA) mutations leading to mitochondrial dysfunction can cause a variety of chronic diseases in central nervous system (CNS). However, the role of mtDNA mutations in sporadic Creutzfeldt–Jakob disease (sCJD) has still been unknown. In this study, we comparatively analyzed complete mtDNA sequences of 31 Chinese sCJD patients and 32 controls. Using MITOMASTER and PhyloTree, we characterized 520 variants in sCJD patients and 507 variants in control by haplogroup and allele frequencies. We classified the mtDNAs into 40 sub-haplogroups of 5 haplogroups, most of them being Asian-specific haplogroups. Haplogroup U, an European-specific haplogroups mtDNA, was found only in sCJD. The analysis to control region (CR) revealed a 31% increase in the frequency of mtDNA CR mutations in sCJD *versus* controls. In functional elements of the mtDNA CR, six CR mutations were in conserved sequence blocks I (CSBI) in sCJD, while only one in control ($P<0.05$). More mutants in transfer ribonucleic acid-Leu (tRNA-Leu) were detected in sCJD. The frequencies of two synonymous amino-acid changes, m.11467A>G, p.(=) in NADH dehydrogenase subunit 4 (ND4) and m.12372G>A, p.(=) in NADH dehydrogenase subunit 5 (ND5), in sCJD patients were higher than that of controls. Our study, for the first time, screened the variations of mtDNA of Chinese sCJD patients and identified some potential disease-related mutations for further investigations.

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INTRODUCTION

Creutzfeldt–Jakob disease (CJD), the most common form of transmissible spongiform encephalopathies (TSEs) or prion disease in human beings, is characterized by the aggregation of partially protease-resistant isoform PrP^{Sc} and spongiform degeneration in the central nervous system (CNS). The most common form of CJD (>85%) is sporadic CJD (sCJD), which occurs with an incidence of about 1–2 per million individuals per year.[1] It is considered to be spontaneous because no epidemiological evidence for association with any exogenous factors.

Although up to now the aetiology of sCJD remains unknown, many researchers have proposed that it has powerful genetic determinants.[2] Studies demonstrate a common polymorphism in the coding region of the PrP gene locus gene (*PRNP*) at codon 129 is a well-established genetic risk factor for sCJD.[3] Homozygosity at *PRNP* codon 129 increases the risk of sCJD in Caucasian.[4] Codons 127 and 219 of *PRNP* also harbour amino-acid (AA) polymorphisms that confer resistance to Kuru[5] or sCJD.[6] Recently, other candidate genes have been identified as risk factors for sCJD, such as an SNP upstream of *PRNP* exon 1 (SNP 1368),[7] c.592C>T(p.T174M) in prion-like doppel gene (*PRND*),[8] *APOE* ε4 allele,[9] polymorphisms at *CALHM1* gene,[10] and *BACE1* gene.[11] Identification of the potential genetic risk factors for sCJD seems to be one of the important pathways for understanding of the pathogenic mechanisms and human susceptibility to the disease.

The mitochondrion is the essential organelle that provides energy in the form of ATP for normal cell function.[12] Although >95% of all enzymes present in the mitochondria are encoded by the nuclear genome, mitochondrial DNA (mtDNA), a 16 569-bp circle of double-stranded DNA in mitochondria contains 37 genes specifying: 13 polypeptides, 22 transfer ribonucleic acids (tRNAs), and 2 ribosomal ribonucleic acids (rRNAs).[13] The rate of sequence evolution in mtDNA is 10–20 times higher than that in the nuclear genome.[14] Thereby mutations in mtDNA are the major reason for the abnormalities of mitochondrion.

Mutations in the mtDNA can result in defect of mitochondrial oxidative phosphorylation (OXPHOS), which may subsequently inhibit ATP production, exacerbate generation of reactive oxygen species (ROS), affect calcium homeostasis, and induce apoptosis.[15] High levels of ROS cause damage of cell membranes through lipid peroxidation and accelerate the high mutation rate of mtDNA. Accumulation of mtDNA mutations enhances oxidative damage, causes energy depletion, and increases ROS production, in a vicious cycle.[16] Moreover, the brain is especially prone to oxidative

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stress-induced damage due to its high levels of polyunsaturated fatty acids, high oxygen consumption, high content in transition metals, and poor antioxidant defenses.[17] There is increasing evidence that impairment of mitochondrial energy metabolism has an important role in the pathophysiology of some chronic brain disorders and the mutations in mtDNA are now recognized as the major contributors to brain disorders, such as Leber's hereditary optic neuropathy (LHON),[18] mitochondrial encephalomyopathy,[19] and Alzheimer's disease (AD).[20] Transition at np 4336 of the mitochondrial tRNA glutamine gene is a risk factor for AD.[21] Since CJD shares similar pathological changes with AD, including abnormal protein deposits in CNS tissues, faulty calcium metabolism, high level of ROS and apoptosis in neurons, it is reasonable to assume a role of mtDNA variants in sCJD.

To date, the situation of mtDNA mutations is rarely described in the patients with sCJD. To explore the potential association of mtDNA mutations or well-known polymorphisms in the control and coding regions with sCJD, a mutational screening of the mtDNA sequences of 31 sCJD patients and 32 non-CJD cases at the ages from 45 to 55 years old was carried out. Analyses of mtDNA sequences in those subjects identified a total of 1131 mtDNA different variants in sCJD patients compared with the revised Cambridge Reference Sequence (rCRS). These variants have been further evaluated by phylogenetic analysis, structure function relation and our efforts were made to establish the relationship of each variant with sCJD.

MATERIALS AND METHODS

Ethics statement

Usage of the stored human peripheral blood samples in China CJD Surveillance System has been approved by the Ethical Committee of National Institute for Viral Disease Prevention and Control, China CDC.

Clinical samples

The peripheral blood samples from 31 Chinese probable sCJD cases and 32 non-CJD cases that did not fulfill the criteria for CJD were included in this study. The diagnoses of CJD were made by China CJD Surveillance Centre according to WHO CJD diagnostic criteria. The diagnosis for each case was made by an expert board consisting of neurologists, neuropathologists, epidemiologists, and laboratory staff. Peripheral blood samples were collected in EDTA-containing vacutainer tubes and stored at $-80\,^{\circ}C$ until DNA extraction. DNA was extracted by using the Qiagen's DNA purification kit (Hilden, Germany) according to the manufacturer's instructions.

PCR Amplification of mtDNA

The whole mitochondrial genome of each tested sample was individually amplified with 24 pairs of primers. PCR amplifications for all primer sets were performed in a 25-μl volume. The PCR products were kept at $-20\,^{\circ}C$ until sequencing. The full mtDNA genome was sequenced. All fragments were sequenced forward and backward for confirmation of any nucleotide variation.

Sequence analysis using MITOMASTER

We assembled the data for both sCJD and non-CJD groups using Jellyfish v 1.3.1 (Field Scientific LLC, Lewisburg, PA, USA), a sequence analysis software in comparison with mtDNA reference, the rCRS (NC_012920.1) provided by the National Center. The complete mtDNA sequence of each case was analyzed by MITOMASTER (http://www.mitomap.org), which included a database of over 3600 mtDNA sequences from NCBI GenBank. Using MITOMASTER, the mtDNA sequence of each case was compared with the rCRS and all mtDNA variants identified. The array of sequence variants was used to determine the haplogroup of the tested case. The haplogroup-associated variants were identified and the population frequency of each nucleotide sequence variant was calculated relative to the MITOMASTER and Pereira's databases. To identify rare variants, we arbitrarily defined variants with allele frequencies $<0.5\%$ as rare and those with frequencies $>0.5\%$ as common, similar to cutoff used for nDNA variation. We have submitted our data to Mitomap database (http://www.mitomap.org/bin/view.pl/MITOMAP/VariantSubmissionList).

Prediction of pathogenicity

For prediction of pathogenic characteristics of all non-synonymous mtDNA changes, two analysis tools were used, Polymorphism Phenotyping v2 (PolyPhen-2) and Sorting Intolerant From Tolerant (SIFT). Polyphen scores of >0.50 are intolerant (possibly damaging). The scores of <0.50 are likely tolerant (benign). SIFT is based on the premise that protein evolution is correlated with protein function.[22,23] SIFT scores range from 0 to 1. The AA substitution is predicted to be damaging if the score is ≤ 0.05, and to be tolerated if the score is >0.05.

Statistical analysis

A case–control association study was carried out between sCJD and non-CJD groups individually for each mtDNA mutation and for Haplogroup. Frequencies between sCJD cases and controls were compared to assess the associations of individual mtDNA mutations and Haplogroup using the Chi-square test. The *P*-value of <0.05 was considered as significant difference. Effect size for the association was measured as an odds ratio (OR) with a 95% confidence interval (CI). A permutation test was employed to address the issue of multiple testing by Bonferroni. The statistical analyses were carried out using the statistical packages Stata SE12.0 (StataCorp LP, College Station, TX, USA).

RESULTS

mtDNA sequence variants detected by sequencing relative to rCRS

mtDNA sequencing following the amplification of whole mtDNA revealed a total of 1131 mtDNA variants in 31 sCJD patients and 1189 mtDNA variants in 32 non-CJD cases compared with the rCRS (Supplementary Table 1). On the basis of both coding- and control-region mutations, including haplogroup nomenclature, mtDNAs from patients and controls were classified into 40 sub-haplogroups of 5 haplogroups followed a phylogenetic tree of global human mtDNA variation. Most of them were typical of modern East Eurasian populations, while a few were West Eurasian (R0 and U) mtDNAs. The only haplogroup that showed statistical association with sCJD patients was haplogroup U, that four sCJD patients, but none of non-CJD cases, belonged to haplogroup U, with a *P*-value of 0.035 (Table 1). However, this significance was not maintained after correcting for multiple hypotheses using a permutation test procedure (adjusted *P*-value = 0.056).

Table 1 Frequencies of mtDNA haplogroups in the groups of sCJD and controls

	sCJD (n = 31)		*Ctrl* (n = 32)					
Haplogroup[a]	n	*%*	n	*%*	*CHI2 exact*	P-*value*	*Adjusted* P-*value*[c]	*OR (95% CI)*
U[b]	4	12.9	0	0	4.409	0.035	0.056	—
R0	1	3.22	1	3.13	0.0005	0.98	1	0.97 (0.06–16.2)
R	4	12.9	10	31.25	1.9846	0.079	0.236	2.36 (0.7–7.96)
N	7	22.58	2	6.25	3.4294	0.064	0.082	0.23 (0.04–1.2)
D	5	16.13	8	25	1.4943	0.38	0.339	2.25 (0.6–8.42)
M	10	32.26	11	34.38	0.0318	0.86	1	1.1 (0.38–3.14)

[a]Haplogroups were grouped according to PhyloTree. U includes U2e and U4b; R0 includes R0 and H1bH; R includes B4d, B5a, F1, F1a, F1b, F3a, and J1c; N includes N9a, A4e, and W3a; D includes D4, D4a, D4b, D4c, D4e, D4i, D4j, D4k, and D5c; M includes M, M1, M7b, M7c, M8a, M9a, C4a, C7, C7a, and Za.
[b]Statistical difference between the groups of sCJD and control with a *P*-value of 0.035, adjusted *P*-value of 0.056.
[c]Adjusted *P*-value: adjustment of Chi-square *P*-values was carried out with a permutation-based approach; number of permutations = 20 000.

Excluding highly polymorphic mtDNA variants in the mtDNA control region (CR) and variants belonged to the mitochondrial haplogroup-specific variants (Supplementary Table 2), the rest of 520 mtDNA variants in 31 sCJD patients, 285 different kinds of mtDNA variants were identified (Supplementary Figure 1).

Novel non-haplogroup-associated mtDNA variants

Haplogroup-associated variation accounted for most of the 520 substitution variants (Supplementary Table 3). On the basis of allele frequencies, there were 36 (13%) novel mtDNA variants. Among them, 13 were mapped in the protein-coding regions. Excluding two variants (m.6228C>T and m.14067C>T) that were haplogroup-associated ones, four variants were non-haplogroup-associated variants leading to synonymous AA changes and seven were non-synonymous variants (Table 2). Four altered AAs out of those seven variants seemed to be able to change the conservative AAs (CI>87%; Table 2). SIFT and PolyPhen analyses proposed that three of them were possibly pathogenic changes. Two variants, m.11375A>C (p.K206Q in NADH dehydrogenase subunit 4 (ND4)) and m.12631T>A (p.S99T in NADH dehydrogenase subunit 5 (ND5)), were observed in the case 9.

Identification and quantification of the mtDNA variants in the CR

To determine the presence of mtDNA mutations in the CR (nps 1_578), the mutants in this region which involved in the known functional elements of the mtDNA CR were selected (Figure 1a). Generally, the frequency of mtDNA CR mutations in the group of sCJD was about 31% increased compared with that of non-CJD (Figure 1b). Regarding the functional elements of the mtDNA CR, there were six CR mutations in conserved sequence blocks I (CSBI) in the sCJD patients, but only one in the non-CJD cases ($P<0.05$), while no obvious difference in the other functional elements was noticed between two groups (Figure 1c). It seems that the group of sCJD patients has more mutations in the functional elements of mtDNA CR, which may influence the biological functions of those elements.

Identification and quantification of the mtDNA variants in the regions for tRNA and rRNA

The mtDNA sequencings identified 40 and 20 nucleotide variations within tRNA genes in the groups of sCJD and non-CJD, respectively (Figure 2). More mutants in tRNA-Leu were observed in the sCJD patients. Especially an A to G transition mutation at position of 12 308, which located in the variable loop of tRNA-Leu, was found in five tested sCJD cases, but not in all non-CJD cases, showing statistical difference in the frequency of m.12308A>G (P-value = 0.018, adjusted P-value = 0.024). The number of mutations within rRNA gene between sCJD and controls were comparable without statistical difference (Supplementary Figure 2).

Case–control association for variations in protein-coding genes

There were 108 variants leading to non-synonymous AA changes in protein coding in those two groups. Frequency of non-synonymous sequence variations in all genes did not reveal statistically significant between sCJD patients and controls (Supplementary Table 4). All nucleotide variations identified in the current study were homoplasmic (Supplementary Table 1).

SIFT and PolyPhen analyses of all non-synonymous changes from sCJD and non-CJD revealed 18 pathogenic changes, without statistical difference between sCJD and non-CJD groups (Supplementary Table 5). Remarkably, two synonymous AA changes c.11467A>G, p.(=) in ND4 and c.12372G>A, p.(=) in ND5 showed higher frequencies in the sCJD patients with statistically different from the non-CJD cases. These two mutants have been reported to be related with altered brain pH value.

DISCUSSION

In prion diseases, the impairment of mitochondrial function is repeatedly observed, which could potentially contribute to or even initiate the various abnormalities, for example, synaptic pathology,[24] faulty calcium metabolism,[25] high level of ROS,[26] and apoptosis of neurons.[27] However, it has still been unclear whether mtDNA mutations as a risk factor for sCJD. In this study, we have screened and compared 31 sCJD cases and 32 controls for mtDNA variations. We have found that haplogroup U appears to be closely associated with Chinese sCJD, though this significance was not maintained after correcting for multiple hypotheses. This finding is somehow unexpected, since European-specific haplogroup U is uncommon in populations of China. The haplogroup-related SNPs may relate to partial uncoupling of OXPHOS and decreased efficiency of ATP production.[28,29] This means that each haplogroup, with its different set of SNPs, can have unique bioenergetic properties and responses to oxidative stressors. The risks of developing PD and AD within the Western European are higher among mtDNA haplogroup H, but lower for haplogroups J and K.[30,31] It is possible that the haplogroup H has significantly higher mitochondrial oxidative damage and higher VO (2max) (oxygen consumption) to produce more ROS.[32] It seems to be that the association between haplogroup U and the risks of sCJD needs further assays in a larger sample scale.

Table 2 Eleven novel non-haplogroup-associated mitochondrial DNA variants in the protein-coding regions

Location/Effect	*Variant*	*Gene*	*AA change*	*CI*	*Haplogroup*	*Case no.*	*Pathogenic*
mRNA/Synon	m.9732C>T	COX1	Silent	0.98	M7b	2	
	m.9974C>T	COX3	Silent	0.98	G1c	15	
	m.11407C>T	ND4	Silent	1	M7b	2	
	m.13821C>T	ND5	Silent	0.07	N9a	10	
mRNA/Nonsyn	m.11375A>C	ND4	p.K206Q	1	N9a	9	Possibly damaging
	m.12473C>T	ND5	p.I46S	0.47	N9a, W3a	9,11	Possibly damaging
	m.12631T>A	ND5	p.S99T	0.93	N9a, W3a	9,11	Possibly damaging
	m.14457T>C	ND6	p.M73V	0.89	U4b	29	Benign
	m.14753C>T	CYTB	p.P3S	0.2	U2e, U2e	21,24	Benign
	m.14980C>A	CYTB	p.I78M	0.4	G1c	15	Benign
	m.15765G>A	CYTB	p.G340E	0.87	B4d	4	Benign

Abbreviations: AA, amino acid; CI, conservation index; COX1, cytochrome c oxidase I; COX3, cytochrome c oxidase III; CYTB, cytochrome B; Nonsyn, non-synonymous; Synon, synonymous.

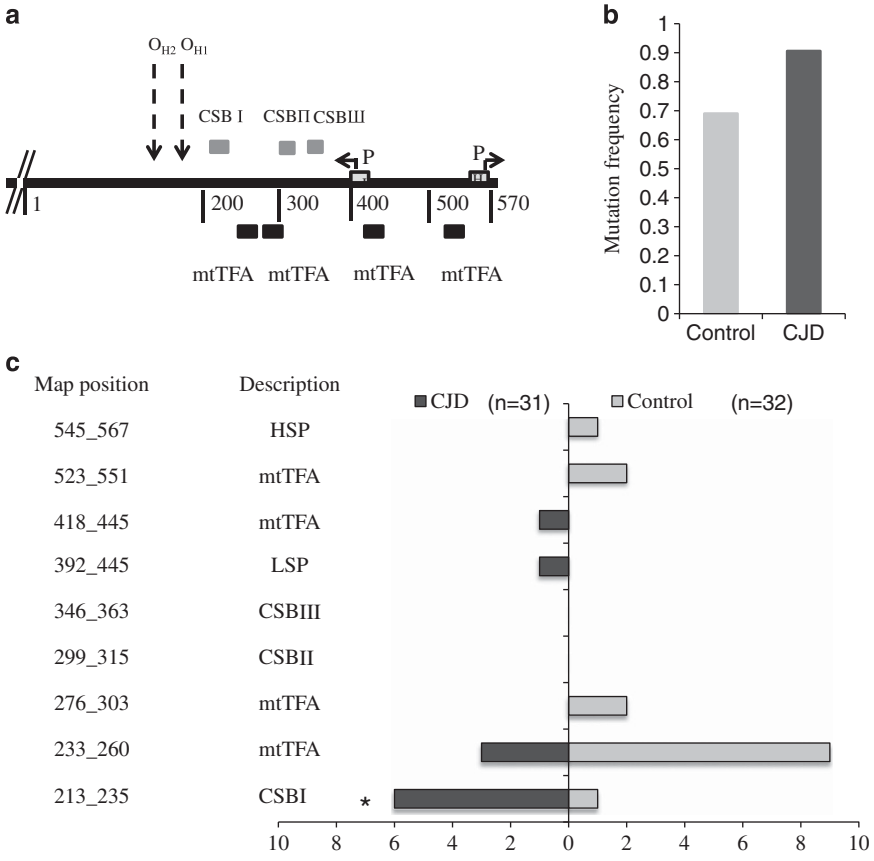

Figure 1 The numbers of the mutations in mtDNA control region (CR) regulatory elements in the groups of sCJD and control. (**a**) Schematic structure of a 570-bp CR in mtDNA. The CR includes the L- and H-strand promoters (PL and PH), the binding sites of mitochondrial transcription factor A (mtTFA), the downstream conserved sequence blocks (CSB) I, II, and III, and the origins of H-strand replication (OH1 and OH2). (**b**) mtDNA CR mutation frequency in the groups of sCJD and control. (**c**) The numbers of heteroplasmic mutations in mtDNA CR regulatory elements in the groups of sCJD and control. *$<$0.05.

On the basis of the review of the relevant literatures and websites, we have found 36 novel mtDNA variants in sCJD patients. Two of them, m.11375A$>$C (p.K206Q in ND4) and m.12631T$>$A (p.S99T in ND5), seem to be pathogenic mutations after assays by a couple of bioinformatics methods. ND4 and ND5 are two of seven subunits of complex I. Studies point out mutations in complex I genes defect in respiration, ATP synthesis, increase ROS production, and are associated with LHON,[33] Leigh's syndrome,[34] mitochondrial encephalomyopathy,[35] lactic acidosis stroke-like episodes (MELAS), and infertility. The exact effect on mitochondrial function of these two mutations deserves further study.

By analyzing the mtDNA CR sequence variation of sCJD and non-CJD, we conclude that sCJD patients display a high frequency of mtDNA CR mutations in the key elements of CSBI. Mutations in CSBI can result in the reduction of mtDNA copy numbers, because the L-strand transcript, processed at CSBI, has been proposed to provide the primer for initiating mtDNA H-strand synthesis at H-strand replication 1 (OH1) and H-strand replication 2 (OH2).[36] Lower mtDNA copy numbers will reduce the amounts of the encoding mitochondrial proteins, and subsequently affect the activities of complexes I, III, IV, and V.

The tRNA-Leu in sCJD patients have more mutations than in non-CJD cases, especially, the mutation of m.12308A$>$G in the variable loop of tRNA-Leu. The tRNA-Leu encodes the most represented AA in the mitochondrial respiratory chain, suggesting a key role of this tRNA in mtDNA-coded OXPHOS subunits. The m.12308A$>$G variation has been reported associated with increased ROS production. The m.12308A$>$G change is a well-known polymorphism defined for European haplogroup U and may constitute a risk factor for occipital stroke in patients with migraine,[37] AD in man,[31] and severe knee osteoarthritis (OA).[38] The association between m.12308A$>$G variation and the risks of sCJD needs further assays in a larger sample scale.

Two synonymous AA changes, m.11467A$>$G in ND4 and m.12372G$>$A in ND5, are more frequently observed in sCJD patients. These two mutants are believed to associate with the pH alteration in brains, which may induce a significantly higher pH value (7.006 ± 0.18 SD) in brain tissues compared with that of control (6.86 ± 0.18 SD).[39] It has been hypothesized that these two mutants lead to loosing coupling due to less excess mitochondrial oxidation and decreased H$+$ ion gradients in the outer membrane. The roles of those two mutations in the pathogenesis of prion diseases remain unsettled.

In summary, our study, for the first time, has screened mtDNA sequence variations in sCJD patients. sCJD patients have more mutations in CSBI *versus* controls. Mutants in tRNA-Leu, especially the frequency of m.12308A$>$G in sCJD patients, have statistical significance. European-specific haplogroup U appears to be closely associated with Chinese sCJD. Two synonymous AA changes, m.11467A$>$G in ND4 and m.12372G$>$A in ND5, show higher frequency in sCJD patients. The exact association between these mtDNA variations and the risks of sCJD needs further detailed assays.

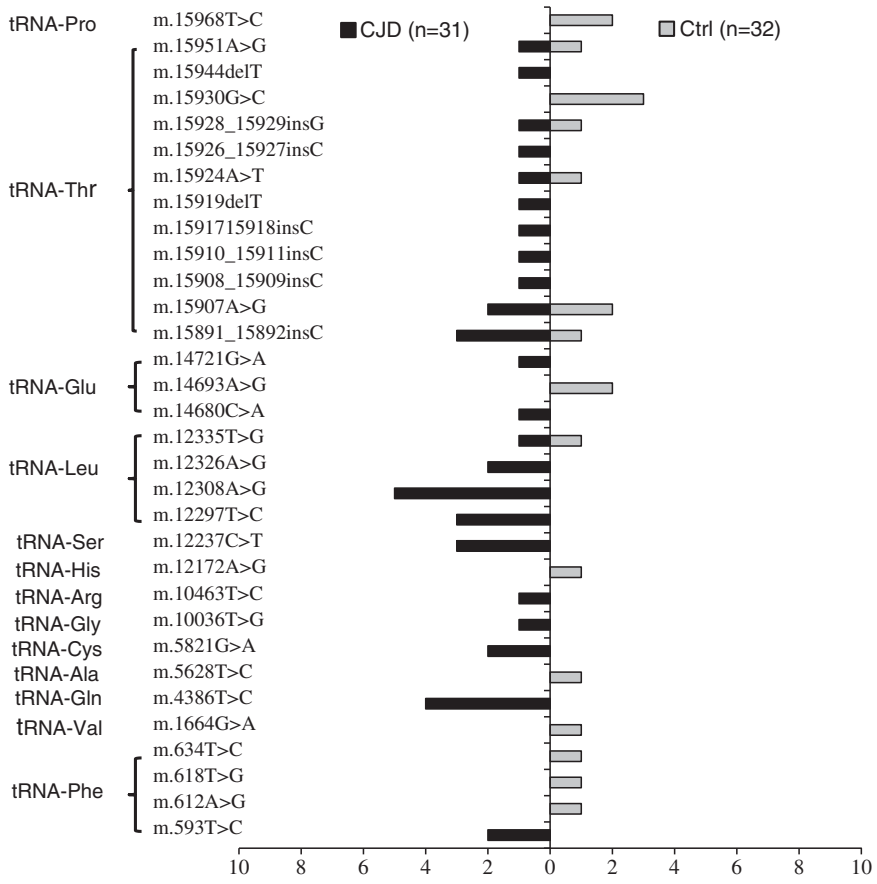

Figure 2 The numbers of the mutations in mtDNA tRNA between the groups of sCJD and controls.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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