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Intra-individual state-dependent comparison of plasma mitochondrial DNA copy number and IL-6 levels in patients with bipolar disorder

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Abstract

Background: Patients with bipolar disorder (BD) have increased plasma IL-6 levels, which are higher in depressed BD (dBD) than remitted BD (rBD). However, the mechanism that differentiates the cytokine levels between dBD and rBD is not understood. First, we determined whether brain-derived mtDNA can be detected in plasma using neuron-specific mutant Polg1 transgenic (Tg) mice. Second, we investigated whether the brain-derived plasma circulating cell-free mitochondrial DNA (ccf-mtDNA) differentiate the cytokine levels between dBD and rBD.

Methods: Mouse plasma ccf-mtDNA levels were measured using real-time PCR targeting two regions of the mtDNA (CO1 and D-loop) in Tg mice and non-Tg littermates. Human plasma ccf-mtDNA levels were measured using real-time PCR targeting two regions of the mtDNA (ND1 and ND4) and IL-6 levels were evaluated in 10 patients in different states (depressed and remitted) of BD in a longitudinal manner and 10 healthy controls.

Results: The mouse plasma CO1/D-loop ratio was significantly lower in Tg than non-Tg mice (P = 0.0029). Human plasma ccf-mtDNA copy number, ND4/ND1 ratio, and IL-6 levels were not significantly different between dBD and rBD. Human plasma ccf-mtDNA levels showed a nominal significant correlation with delusional symptoms (P = 0.033, ρ = 0.68).
Limitations: A larger sample size is required to generalize the results and to determine whether plasma ccf-mtDNA is associated with systemic inflammation.

Conclusions: Tg mice revealed that brain-derived mtDNA could be present in peripheral blood. The present findings did not coincide with our hypothesis that plasma ccf-mtDNA differentiates the cytokine levels between dBD and rBD.

Keywords: bipolar disorder, circulating cell-free mitochondrial DNA, damage-associated molecular patterns, interleukin-6.
Introduction

Bipolar disorder (BD) is a mood disorder that causes depressive and (hypo)manic symptoms. The biology of BD is not completely understood, but evidence for the involvement of inflammation in both the peripheral and central nervous systems has been accumulating over time (Benedetti et al., 2020; Giridharan et al., 2020). An increase in the levels of the inflammatory cytokine, IL-6, is most frequently reported in the plasma of patients with BD (Köhler et al., 2017). Plasma IL-6 levels are higher in depressed patients with BD (dBD) than that in patients with remitted BD (rBD) (Brietzke et al., 2009). However, the mechanism of this differentiation in cytokine levels between the dBD and rBD is not understood.

Recently, circulating cell-free mitochondrial DNA (ccf-mtDNA) has received attention as it provides a potential link between the brain and immune system in neuroimmunological disorders (Gambardella et al., 2019) such as BD (Benedetti et al., 2020). Mitochondrial dysfunction in the brain is implicated in BD (Kasahara and Kato, 2018; Kato, 2017; Kato and Kato, 2000; Scaini et al., 2021). Mitochondrial dysfunction triggers the release of a variety of damage-associated molecular patterns (DAMPs), which cause innate inflammatory responses (Cruz and Kang, 2018). MtDNA plays a role as a DAMP as the CpG sites of mtDNA are not methylated, similar to those in bacterial
DNA. Toll-like receptor 9 recognizes the non-methylated CpG sites of mtDNA and activates the interferon gene pathway and the production of inflammatory cytokines and chemokines including IL-6 (Klinman et al., 1996; Takeshita et al., 2001; Zhang et al., 2010). Recent studies have demonstrated that interactions between the immune system and the brain can lead to changes in mood, cognition, and behavior (Dantzer et al., 2008; Pape et al., 2019). Regarding the structural neuroimaging studies in BD, the key finding is decreased subcortical regional volumes, especially in the amygdala and hippocampus (Phillips et al., 2014). Studies have reported decreased amygdala volume in adults with bipolar disorder, particularly during depressive episodes compared to the remitted state (Foland-Ross et al., 2012). For that reason, the amount of mtDNA released from the decreased brain to the peripheral blood flow may be different between dBD and rBD. Thus, it is possible that plasma ccf-mtDNA, derived from the brain, is associated with the elevation of systemic cytokines as a DAMP (Kageyama et al., 2018), and with the differing plasma cytokine levels between dBD and rBD.

To answer this question, we first investigated whether mtDNA released from the mitochondria in the brain could circulate in the peripheral blood. Two rationales support the hypothesis that brain-derived mtDNA can cross the blood-brain barrier: neuroinflammation and exosomes. Neuroinflammation induces the leakage of the
blood-brain barrier. During this time, mtDNA released from damaged neurons crosses the ependymal wall to reach the cerebrospinal fluid and blood, wherein it is detectable as biomarkers of neuroinflammation (Gambardella et al., 2019). As an alternative pathway, mtDNA in the neural cells is packed inside exosomes, which then cross the blood-brain-barrier and are released into the peripheral blood stream (Hornung et al., 2020; Picca et al., 2019; Sansone et al., 2017). However, it is still unclear whether plasma ccf-mtDNA includes brain-derived mtDNA because it is difficult to distinguish the tissue of origin of ccf-mtDNA. To solve this problem, we used transgenic mice expressing mutant Polg driven by the CaMKIIα promoter, in whom deleted mtDNA are produced only in the neurons of the forebrain (Bagge et al., 2020; Kasahara et al., 2016, 2006). By measuring the deleted mtDNA, we could evaluate whether brain-derived mtDNA is present in the peripheral blood.

We proposed two hypotheses: (1) plasma ccf-mtDNA, inflammatory cytokine, and mitochondrial dysfunction levels differ in a state-dependent manner in BD, and (2) these levels are related to the severity of the clinical symptoms. To evaluate mitochondrial dysfunction, we focused on a 4,977-bp common deletion, which is related to mitochondrial dysfunction (Zhang et al., 2015). Human mtDNA is a 16,569 bp circular molecule. The 4,977-bp common deletion eliminated between nucleotides 8470 and
13447 of the human mitochondrial genome, including five tRNA genes and seven genes (ND3, ND4, ND4L, partial ND5, COX III, ATP6, and partial ATP8) (Mohamed Yusoff et al., 2019). Increased levels of the 4,977-bp common deletion have been reported in autopsied brains of patients with BD (Kato et al., 1997). To investigate our hypothesis, we measured the plasma ccf-mtDNA level, the ND4/ND1 ratio – as an indicator of 4,977-bp common deletion (He et al., 2002; Kakiuchi et al., 2005) – and the IL-6 levels during the depressed and remitted states in the same patient with BD in a longitudinal manner.

Here, we provide evidence that the brain-derived mtDNA could be present in the peripheral blood by using the mutant mice. Human plasma ccf-mtDNA copy number, ND4/ND1 ratio, and IL-6 levels were not significantly different between dBD and rBD. The plasma ccf-mtDNA level nominally showed a positive correlation with delusional symptoms in patients with dBD.

Materials and methods

Animals

All mutant Polg1 transgenic (Tg) mice were heterozygous. Male Tg mice were mated with wild-type C57BL/6J female mice. Tg mice express a proofreading-deficient Polg1
transgene only in the forebrain neurons by the CaMKIIα promoter at expression levels comparable to endogenous Polg1, and deleted mtDNAs are produced and accumulated only in the brain (Kasahara et al., 2016, 2006).

Heterozygous female Tg and non-Tg littermates (55–59 weeks, n = 12 for each condition) were euthanized using carbon dioxide. Blood samples were immediately withdrawn through an abdominal aorta puncture. The blood was centrifuged at 3,000 × g for 15 min, and the plasma was kept frozen at -80 °C until assayed.

Quantification of mouse plasma ccf-mtDNA copy number

Mouse plasma ccf-DNA was extracted from 50 μL of plasma using a QIAamp DNA Micro Kit (Qiagen, Hilden, Germany) and eluted with 50 μL of deionized distilled water. The eluent contained ccf-mtDNA and ccf-DNA derived from nuclear chromosomes.

In ccf-mtDNA, we assessed the amount of deleted mtDNA by real-time qPCR using a QuantiTect Multiplex PCR Kit (Qiagen) according to the manufacturer’s instructions. The amount of deleted mtDNAs were determined from the copy number ratio of two mtDNA segments, the CO1 and D-loop regions, which were lost and preserved, respectively, in deleted mtDNA. The PCR primers and TaqMan MGB probes (Thermo Fisher Scientific, MA, USA) used in the assay are as follows: for CO1, forward
primer 5′-AACCCCCAGCCATAACACAG-3′, reverse primer
5′-GTATAGTAATGCCTGCGGCTAGC-3′, probe (FAM-dye labeled)
5′-CCGTACTGCTCCTAT-3′; for D-loop (control region), forward primer
5′-CCCTCCTCTTTAATGCCCAAAC-3′, reverse primer
5′-TGATCAGGACATAGGGTTTGATAG-3′, probe (VIC-dye labeled)
5′-AACACTAAGAACTTGAAAGAC-3′.

The absolute concentration of plasma ccf-mtDNA was estimated according to
the standard curves derived from the known concentration of plasmid DNA containing
CO1 and D-loop regions. Values are expressed as plasmid copy number per microliter of
plasma.

The plasmid DNA construct used for the standard was generated as follows. The
D-loop region (83 bp) of mouse mtDNA was amplified using PfuUltra High Fidelity
DNA Polymerase (Agilent Technologies, CA, USA) with the forward primer 5′-
CCCTCCTCTTTAATGCCCAAAC -3′ and reverse primer 5′-
TGATCAGGACATAGGGTTTGATAG -3′. The amplicon was inserted into the
pCR-Blunt II-TOPO vector (Thermo Fisher Scientific). The CO1 region (111 bp) was
amplified with the forward primer 5′- AACCCCCAGCCATAACACAG -3′ and the
reverse primer 5′ - GTATAGTAATGCCTGCGGCTAGC -3′. The D-loop/pCR-Blunt II
plasmid was cut with EcoRV and ligated with the PCR product of CO1 using a DNA Ligation Kit, Mighty Mix (Takara Bio, Shiga, Japan). The resultant plasmid (3,713 bp) was verified as having only a single copy of each insert using Sanger sequencing. The plasmid DNA was purified using the PureLink HiPure Plasmid Midiprep Kit (Thermo Fisher Scientific). The plasmid DNA copy number was calculated based on ultraviolet absorbance, which was measured using a NanoDrop 1000 (Thermo Fisher Scientific).

Participants

Human plasma samples of patients with BD ($n = 10$) were collected during both dBd and rBD states from the same individuals in a longitudinal manner derived from participants at Osaka City University Hospital. Control samples ($n = 10$) were obtained from individuals recruited from among the healthy spouses of patients and hospital staff in the Osaka City University Hospital and Hannan Hospital. These patients and healthy controls were different subjects from previously reported (Kageyama et al., 2018).

Trained psychologists or psychiatrists conducted a structured interview using the Mini-International Neuropsychiatric Interview (M.I.N.I.), Japanese version, for all participants. A diagnosis was made according to the criteria listed in the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV). Patients with any
comorbid axis I disorder, severe head injury, substance abuse/dependence, or a prior medical history of central nervous system disease were excluded from the study. Depressive symptoms were assessed using the Japanese version of the 21-item Hamilton Depression Rating Scale (HAMD-21). HAMD items were pooled into the following categories: ‘core’ (items 1, 2, 7, 8, 10, and 13), ‘sleep’ (items 4, 5, and 6), ‘activity’ (items 7 and 8), ‘psychic anxiety’ (items 9 and 10), ‘somatic anxiety’ (items 11, 12, and 13), and ‘delusion’ (items 2, 15, and 20) (Bellini et al., 1992; Lattuada et al., 1999). We divided the patients into dBD and rBD based on the results of the M.I.N.I. (Sheehan et al., 1998). We defined dBD as past manic episode with a current depressive episode. We defined rBD as past manic episode without a current depressive or manic episode. The M.I.N.I could properly differentiate the status of BD patients (Sheehan et al., 1998). The patients received any mood stabilizer(s) (lithium carbonate, sodium valproate, carbamazepine, and lamotrigine) with or without antipsychotics. The dose of daily antipsychotics was estimated using chlorpromazine equivalents. No one received any anxiolytic or hypnotic.

**Blood sampling**
Blood samples were withdrawn from each subject through vein puncture and immediately centrifuged at 3,000 \( \times \) g for 15 min. The plasma was stored at -80 °C until assayed.

**Ethics statement**

After the nature of the study procedures was fully explained, all participants provided written informed consent. This study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Osaka City University and the Ethics Committee of Hannan Hospital. The Wako Animal Experiment Committee, RIKEN, approved all the animal experimental protocols.

**Demographic characteristics of study participants**

No significant differences in age or sex ratios were observed between the groups. All the HAMD-21 score and other scores of the subcategories (core, sleep, activity, psychic anxiety, somatic anxiety and delusion) were significantly higher in dBD compared to rBD (HAMD-21, \( P = 0.0020 \); core, \( P = 0.0020 \); sleep, \( P = 0.0020 \); activity, \( P = 0.0020 \); psychic anxiety, \( P = 0.0039 \); somatic anxiety, \( P = 0.0020 \); delusion, \( P = 0.0039 \)). No significant difference was seen between dBD and rBD regarding to the medication dose.
(anti psychotant, \( P = 0.25 \); lithium carbonate, \( P = 0.50 \); sodium valproate, \( P = 0.38 \); lamotrigine, \( P = 0.99 \)) (Table 1). The gap of the day of collecting samples between dB and rBD was 371.4 \( \pm \) 290.9 days.

**Quantification of human plasma ccf-mtDNA copy number**

The details of the methods used for the quantification of human plasma ccf-mtDNA copy numbers have been described previously (Kageyama et al., 2018). The amount of deleted mtDNAs were determined from the copy number ratio of two mtDNA segments, the \( ND4 \) and \( ND1 \) regions, which were lost and preserved, respectively, in the 4,977-bp common deletion of the human mtDNA (He et al., 2002).

**Cytokine measurements**

Human plasma levels of IL-6 were measured using the Human IL-6 Quantikine HS ELISA Kit (HS600C; R&D Systems, MN, USA) according to the manufacturer’s instructions. The assays were performed using the Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific). Values are expressed as pg/mL.

**Statistical analysis**
Data are presented as means ± standard deviation (SD). Categorical variables were compared using the chi-squared test. For comparison for dBD and rBD, Wilcoxon matched-pairs signed rank test was used. For comparison of age between BD and control, and plasma CO1/D-loop ratio between mutant Polg1 Tg mice and non-Tg littermates, Mann-Whitney U test was used. Correlation coefficients was calculated using Spearman’s rank correlation coefficients. For a comparison of dBD, rBD and control groups, a Kruskal-Wallis test followed by multiple comparisons with Steel-Dwass test was used. Statistical significance was set at $P < 0.05$. As for the correlation analysis between the ccf-mtDNA copy number and the clinical assessment in dBD, multiple comparisons were corrected using the Bonferroni correction. The effect size was calculated using the Cliff's Delta statistic which is a non-parametric effect size measure for Wilcoxon matched-pairs signed rank test and Mann-Whitney U test. Post hoc power analysis for the comparison of ND1 between dBD and rBD was calculated using G-Power version 3.1, wherein we used Wilcoxon signed-rank test (matched pairs) and regarded the value of power $(1 - \beta)$ as 0.90, $\alpha$ error = 0.05, two-tails and parent distribution as logistic distribution. Finally, statistical analyses were performed using the IBM SPSS Statistics Version 24, Japanese version (IBM Japan, Tokyo, Japan), R version 3.6.3
(http://cran.r-project.org/) and the Graph-Pad Prism 8.3.1 statistical program (GraphPad Software Inc., CA, USA).

**Results**

**Detection of the brain-derived deleted mtDNA in peripheral circulation**

We compared the plasma $CO1/D$-loop ratio, which is an indicator of deleted mtDNA and $D$-loop-containing multimers that accumulated only in the brain of Tg mice (Bagge et al., 2020; Kasahara et al., 2006). A schematic diagram of the full-length mouse mtDNA is shown in Figure 1a. The ratio was significantly lower in Tg mice ($0.64 \pm 0.01$) than that in non-Tg mice ($0.66 \pm 0.01$, $P = 0.0029$, effect size of Cliff’s Delta = 0.69, Figure 1b).

There was no significant correlation between ccf-mtDNA levels measured by the $D$-loop region and the mtDNA deletion levels assessed by the $CO1/D$-loop ratio in Tg ($P = 0.72$, $\rho = -0.12$) or non-Tg mice ($P = 0.24$, $\rho = -0.37$) using Spearman’s rank correlation coefficients. The small $CO1/D$-loop ratio indicates that brain-derived mtDNA is present in the plasma. But the no significant correlation between the ccf-mtDNA level and $CO1/D$-loop indicates that mtDNA from tissues other than the brain are also contained in the plasma.
Intra-individual comparison of the plasma mtDNA copy number and IL-6 levels in patients with BD

First, we confirmed a strong significant correlation between the copy number of the two regions of the mtDNA i.e., ND1 and ND4 \((P < 0.0001, \rho = 0.99, \text{Supplementary Figure S1})\). Thus, we used the ND1 copy number as a representative value of the mtDNA copy number.

Next, we compared the plasma ccf-mtDNA copy number, IL-6 level, and ND4/ND1 ratio between rBD and dBD of the same patients using a Wilcoxon matched-pairs signed rank test. There was no significant difference in the ND1 copy number between rBD \((1.03 \times 10^4 \pm 0.69 \times 10^4 \text{ copies/\mu L})\) and dBD \((5.00 \times 10^4 \pm 7.83 \times 10^4 \text{ copies/\mu L}, P = 0.065\), effect size of Cliff's Delta = 0.42, Figure 2a). There was no significant difference in the ND4/ND1 ratio between rBD \((0.83 \pm 0.05)\) and dBD \((0.85 \pm 0.07, P = 0.85, \text{effect size of Cliff's Delta} = 0.10, \text{Figure 2b})\). IL-6 levels did not show a significant difference between rBD \((1.3 \pm 0.7 \text{ pg/mL})\) and dBD \((1.2 \pm 0.7 \text{ pg/mL}, P = 0.63, \text{effect size of Cliff's Delta} = 0.10; \text{Figure 2c})\). We conducted a Kruskal-Wallis test followed by multiple comparisons with Steel-Dwass test for a comparison of dBD, rBD and control groups. Plasma mtDNA copy number did not differ among 3 groups (control; \(6.99 \times 10^4 \pm 12.4 \times 10^4 \text{ copies/\mu L}, P = 0.42\)). ND4/ND1 ratio did not differ among 3 groups (control; \(0.81 \pm 0.042, P = 0.51\)).
IL-6 level was significantly higher in rBD compared to control group (0.61 ± 0.20 pg/mL, 
\( P = 0.014 \)).

**Relationship between the ccf-mtDNA copy number and the clinical assessment in dBD**

To investigate the relationship between the plasma ccf-mtDNA copy number and patients’ symptoms (HAMD-21, HAMD core, HAMD sleep, HAMD activity, HAMD psychic anxiety, and HAMD delusion), we conducted a correlation analysis using Spearman’s rank correlation coefficients (Table 2).

A nominal, significant, positive correlation was seen between the plasma ccf-mtDNA levels and HAMD-21 subscale of ‘delusion’ score (\( P = 0.033, \rho = 0.68 \)) (Supplementary Figure S2). However, the result was not significant after the Bonferroni collection.

**Correlation between plasma ccf-mtDNA and IL-6 levels in dBD**

There was no significant correlation between plasma ccf-mtDNA levels and plasma IL-6 
levels in dBD (\( P = 0.73, \rho = -0.13 \)) (Supplementary Figure S3).

**Medication effects on the plasma ccf-mtDNA copy number in dBD**
We suspected that medication might affect the plasma ccf-mtDNA copy number. To examine the effects of medication on the plasma ccf-mtDNA copy number, we divided the patients into two subgroups: medicated or drug-free. As only one dBD and no rBD patients were drug-free, we could not compare the two subgroups. Next, we conducted a correlation analysis using Spearman’s rank correlation coefficients between the plasma ccf-mtDNA copy number and each mood stabilizer administered in dBD (Table 3), rBD (Table 4), and dBD plus rBD (Supplementary Table S1). A significant correlation was observed between plasma ccf-mtDNA levels and lamotrigine levels in patients with dBD ($P = 0.015, \rho = -0.99$) (Table 3). The statistical significance survived after the Bonferroni collection. Although there was no significant correlation between plasma ccf-mtDNA levels and lamotrigine in rBD ($P = 0.29, \rho = -0.9$) (Table 4) or dBD plus rBD ($P = 0.39, \rho = 0.015$) (Supplementary Table S1). To determine whether lamotrigine intake affects the plasma ccf-mtDNA copy number, we compared the plasma ccf-mtDNA copy number between patients with BD who were administered lamotrigine and those who were not. The plasma ccf-mtDNA copy number was higher in dBD with lamotrigine ($13.5 \times 10^4 \pm 10.9 \times 10^4$ copies/μL) compared to that in dBD without lamotrigine ($1.37 \times 10^4 \pm 1.20 \times 10^4$ copies/μL, $P = 0.013$). However, there was no significant difference in the plasma mtDNA copy number between rBD patients who were administered lamotrigine
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(1.39 ×10^4 ± 0.80 ×10^4 copies/μL) and rBD patients who were not (0.88 × 10^4 ± 0.64 ×10^4 copies/μL, P = 0.32).

Discussion

To the best of our knowledge, this is the first report describing the presence of brain-derived mtDNA in plasma using a mouse model, and the relationship between plasma ccf-mtDNA level, IL-6 level, and clinical symptoms in BD patients in a longitudinal manner.

We found that the plasma CO1/D-loop ratio was lower in Tg mice compared to that in non-Tg mice (Figure 2b). This indicates a higher level of deleted mtDNA and D-loop-containing multimers of mtDNA in the plasma of Tg mice than that in non-Tg mice. Kasahara et al. showed that the deleted mtDNAs are present only in the forebrain of Tg mice and were absent in other tissues and in non-Tg mice (Kasahara et al., 2006). Bagge et al. found that Tg mice induced the accumulation of D-loop-containing multimers of mtDNA in the brain. It should be noted that deleted mtDNAs are also synthesized from the peripheral organs due to the effect of aging (Bagge et al., 2020); however, the smaller value of the CO1/D-loop ratio in the plasma of brain-specific Tg
mice strongly suggests that deleted mtDNA and multimers flowed from the brain to the peripheral circulation.

We did not detect a significant difference in the plasma ccf-mtDNA copy number between the rBD and dBD in the same individual, although the effect size showed a medium effect (Cliff’s Delta = 0.42). We computed the post hoc power analysis using the G-Power software with sufficient statistical power. Only a difference with an effect size of 1.09 can be detected with Wilcoxon signed-rank test (matched pairs) wherein we regarded $n = 10$, the value of power $(1 – \beta)$ as 0.90, $\alpha$ error = 0.05, two-tails and parent distribution as logistic distribution. Thus, more subtle differences can be overlooked in this sample size, and we re-estimated the required sample size using Cliff’s Delta = 0.42, power $(1 – \beta) = 0.90$, and $\alpha$ error = 0.05. The required sample size was $n = 110$, which suggests that our result might be due to type II error due to the small sample size. Plasma ccf-mtDNA is reportedly affected by exercise (Lim et al., 2000) possibly due to its release from muscles (Butt and Swaminathan, 2008). Decreased physical activity during the depressed state could have decreased the circulating mtDNA (Kageyama et al., 2018), which could be a confounding factor. Recently, a novel method for isolating neuron-derived exosomes from plasma has been investigated (Nasca et al., 2020). This method enables us to indirectly evaluate brain-origin mtDNA that are packed in
neuron-derived exosomes. This should be a good solution to avoid the confounding factors. In this study, the \( ND4/ND1 \) ratio had no significant state-dependent differences in BD patients. The common deletion is related not only to mitochondrial dysfunction but also to cancer (Mohamed Yusoff et al., 2019), Alzheimer’s disease (Casoli et al., 2015), coronary atherosclerotic heart disease (Corral-Debrinski et al., 1992), and aging (Lee et al., 1994). These possible confounding factors might weaken the relationship between mitochondrial dysfunction and the \( ND4/ND1 \) ratio.

Plasma ccf-mtDNA levels had no significant correlation with IL-6 levels in BD, which was a similar result to that previously reported in patients with major depressive disorder (Kageyama et al., 2018). In the plasma, mtDNA activates toll-like receptor 9 on dendritic cells, which activates the helper T-cell-mediated signaling pathway in circulating neutrophils, resulting in increased production of pro-inflammatory mediators such as IL-6 (Grazioli and Pugin, 2018). Proper IL-6 expression is strictly controlled, such as chromatin structure, transcriptional regulation, and post-transcriptional modification. The differentiation status of cells, various transcription factors, RNA-binding proteins, and microRNA also influence the process (Tanaka et al., 2014). Mitochondrial dysfunction induces the release of DAMPs, such as mtDNA, \( N \)-formyl peptides, cytochrome c, and cardiolipin. These molecules induce complex immune
reactions; thus, mtDNA itself may not have a clear correlation with IL-6 levels. Another possibility is that the patient population in this study was different from that in previous studies in which altered cytokine levels were reported. A certain population of BD with elevated plasma cytokines should be tested to determine whether plasma ccf-mtDNA contributes to the elevation of cytokines in BD.

We found that the plasma ccf-mtDNA level showed a nominal positive correlation with delusional symptoms in patients with dBD. Importantly, delusional symptoms are significantly associated with neuroinflammation (Schiavone and Trabace, 2017), which is known to be associated with ccf-mtDNA (Gambardella et al., 2019). Taking these and our results into consideration, plasma ccf-mtDNA might have a potential link with delusional symptoms. However, we need to verify the reproducibility of the correlation between plasma ccf-mtDNA and delusional symptoms in patients with dBD in an independent sample set. A previous study showed that whole blood mtDNA copy number is negatively associated with the severity of psychosis in patients with BD and schizophrenia (Kumar et al., 2018). Notably, whole blood mtDNA originates mainly from leukocytes and platelets (Hurtado-Roca et al., 2016; Urata et al., 2008), and its significance is completely different from that of ccf-mtDNA. Thus, our results and the previous study are not comparable with each other.
The plasma ccf-mtDNA copy number was higher in patients with dBD who were administered lamotrigine compared to that in dBD patients who were not \((P = 0.013)\). Lamotrigine has not only a neuroprotective effect but also a mitochondrial toxicity effect (Finsterer and Scorza, 2017) as it causes the attenuation of proteasome inhibition-induced apoptosis by suppressing the activation of the mitochondrial pathway and the Caspase-8- and Bid-dependent pathways (Nam et al., 2016). This mitochondrial toxicity effect might contribute to the plasma ccf-mtDNA copy number. However, there was no significant difference in the plasma mtDNA copy number between rBD with lamotrigine and rBD without lamotrigine \((P = 0.32)\). These data suggested that the plasma ccf-mtDNA copy number might be affected by the mood state rather than by the administration of lamotrigine itself. Therefore, we must be careful in interpreting the plasma ccf-mtDNA copy number difference between patients with dBD who were administered lamotrigine and those who were not.

**Limitations**

There were several limitations to the present study. First, the number of subjects was too small to draw a satisfactory conclusion. A larger sample size is needed to determine
whether plasma ccf-mtDNA shows a state-dependent change in patients with BD. Second, we showed the results with only one sample set. For further study, we need to confirm the reproducibility of our results with an independent sample set. Third, we collected the samples from 9 am to 12 pm without fasting. It cannot be excluded that the circadian validation (Sardon Puig et al., 2018; Wang et al., 2020) and diet (Lam and McKeague, 2019) affected plasma mtDNA copy number. Because we did not strictly control the sample collection time and the meals, it cannot be ruled out that the observed changes can be ascribed to these confounding factors. Finally, the quantity ratio of plasma mtDNA comes from the brain origin, and another origin is unknown.

Conclusions

Using a mouse model, we found that ccf-plasma mtDNA could include brain origin mtDNA. Plasma mtDNA copy number, IL-6 level, and ND4/ND1 ratio did not significantly differ between the rBD and dBD in the same individual. The plasma ccf-mtDNA level showed a nominal positive correlation with delusional symptoms in patients in the dBD. Further studies are needed to explore the biological significance of the relationship between mtDNA levels and inflammation by focusing on neuron-derived exosomes in plasma samples.
Declarations of interest

All authors declare that they have no conflict of interest relevant to this study.

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Author Contributions

YK conceived and designed the experiments. YK performed all molecular biological experiments and analyzed the data. T Kasahara supervised all molecular biological experiments. YK, YD, MT, KK and KI collected human samples and drafted the manuscript. YK, YD, T Kasahara, and T Kato wrote the paper. All authors read and approved the final manuscript.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.
Acknowledgments

We thank the patients and their families who participated in our study, and Dr. Taku Doi and Dr. Mariko Yamada, who assisted us in sample collection.
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Table 1. Demographic and clinical characteristics of participants with bipolar disorder and of controls.

<table>
<thead>
<tr>
<th></th>
<th>Bipolar disorder</th>
<th>Control</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Remitted</td>
<td>Depressed</td>
<td></td>
</tr>
<tr>
<td>n (Male/Female)</td>
<td>10 (2/8)</td>
<td>10 (2/8)</td>
<td>1*</td>
</tr>
<tr>
<td>Age, years</td>
<td>46.5 ± 16.8</td>
<td>47.6 ± 15.5</td>
<td>0.75b</td>
</tr>
<tr>
<td>HAMD-21, score</td>
<td>2.7 ± 1.3</td>
<td>22.9 ± 4.5</td>
<td>N.A.</td>
</tr>
<tr>
<td>HAMD core, score</td>
<td>1.6 ± 0.9</td>
<td>10.3 ± 2.1</td>
<td>N.A.</td>
</tr>
<tr>
<td>HAMD sleep, score</td>
<td>0.7 ± 0.8</td>
<td>3.8 ± 1.1</td>
<td>N.A.</td>
</tr>
<tr>
<td>HAMD activity, score</td>
<td>0.1 ± 0.3</td>
<td>4.2 ± 1.4</td>
<td>N.A.</td>
</tr>
<tr>
<td>HAMD psychic anxiety, score</td>
<td>0.8 ± 0.9</td>
<td>3.5 ± 1.3</td>
<td>N.A.</td>
</tr>
<tr>
<td>HAMD somatic anxiety, score</td>
<td>0.4 ± 0.5</td>
<td>3.5 ± 1.6</td>
<td>N.A.</td>
</tr>
<tr>
<td>HAMD delusion, score</td>
<td>0</td>
<td>2.3 ± 1.2</td>
<td>N.A.</td>
</tr>
<tr>
<td>anti psychotant, mg/day (ratio)</td>
<td>85.1 ± 208.1 (2/10)</td>
<td>127.7 ± 202.2 (5/10)</td>
<td>N.A.</td>
</tr>
<tr>
<td>Lithium carbonate, mg/day (ratio)</td>
<td>240 ± 263.3 (5/10)</td>
<td>190 ± 251.4 (4/10)</td>
<td>N.A.</td>
</tr>
<tr>
<td>Sodium valproate, mg/day (ratio)</td>
<td>460 ± 313.4 (8/10)</td>
<td>360 ± 362.7 (6/10)</td>
<td>N.A.</td>
</tr>
<tr>
<td>Carbamazepine, mg/day (ratio)</td>
<td>30 ± 94.9 (1/10)</td>
<td>30 ± 94.9 (1/10)</td>
<td>N.A.</td>
</tr>
<tr>
<td>Lamotrigine, mg/day (ratio)</td>
<td>55 ± 106.5 (3/10)</td>
<td>80 ± 131.7 (3/10)</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

*Chi-squared test.

*Mann-Whitney U test.

*Wilcoxon matched-pairs signed rank test.

Abbreviations: HAMD-21, 21-item Hamilton Depression Rating Scale; N.A., not applicable.
Table 2. Relationship between the plasma ccf-mtDNA copy number and the clinical assessments in dBD

<table>
<thead>
<tr>
<th></th>
<th>$\rho$</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAMD-21, score</td>
<td>0.55</td>
<td>0.10a</td>
</tr>
<tr>
<td>HAMD core, score</td>
<td>0.43</td>
<td>0.21a</td>
</tr>
<tr>
<td>HAMD sleep, score</td>
<td>0.23</td>
<td>0.56a</td>
</tr>
<tr>
<td>HAMD activity, score</td>
<td>0.0065</td>
<td>0.99a</td>
</tr>
<tr>
<td>HAMD psychic anxiety, score</td>
<td>0.55</td>
<td>0.11a</td>
</tr>
<tr>
<td>HAMD somatic anxiety, score</td>
<td>0.16</td>
<td>0.66a</td>
</tr>
<tr>
<td>HAMD delusion, score</td>
<td>0.68</td>
<td>0.033a</td>
</tr>
</tbody>
</table>

*Spearman’s rank correlation analyses

Abbreviations: dBD, depressed state of bipolar disorder; HAMD-21, 21-item Hamilton Depression Rating Scale.
Table 3. Correlation between the plasma ccf-mtDNA copy number and medications in dBD

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>$\rho$</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lithium carbonate, mg/day (ratio)</td>
<td>4</td>
<td>0.47</td>
<td>0.53$^a$</td>
</tr>
<tr>
<td>Sodium valproate, mg/day (ratio)</td>
<td>6</td>
<td>-0.48</td>
<td>0.33$^a$</td>
</tr>
<tr>
<td>Carbamazepine, mg/day (ratio)</td>
<td>1</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>Lamotrigine, mg/day (ratio)</td>
<td>3</td>
<td>-0.99</td>
<td>0.015$^a$</td>
</tr>
</tbody>
</table>

$^a$Spearman’s rank correlation analyses

Abbreviations: dBD, depressed state of bipolar disorder; N.A., not applicable.
Table 4. Correlation between the plasma ccf-mtDNA copy number and medication in rBD

<table>
<thead>
<tr>
<th>Medication</th>
<th>n</th>
<th>ρ</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lithium carbonate, mg/day (ratio)</td>
<td>5</td>
<td>0.41</td>
<td>0.50$^a$</td>
</tr>
<tr>
<td>Sodium valproate, mg/day (ratio)</td>
<td>8</td>
<td>0.044</td>
<td>0.91$^a$</td>
</tr>
<tr>
<td>Carbamazepine, mg/day (ratio)</td>
<td>1</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>Lamotrigine, mg/day (ratio)</td>
<td>3</td>
<td>-0.90</td>
<td>0.29$^a$</td>
</tr>
</tbody>
</table>

$^a$Spearman’s rank correlation analyses

Abbreviations: rBD, remitted state of bipolar disorder; N.A., not applicable.
Figure Legends

Figure 1.

**Plasma deleted mtDNA in mutant Polg1 transgenic (Tg) mice.**

a) A schematic diagram of a full-length mouse mtDNA. b) Comparison of plasma CO1/D-loop ratio between mutant Polg1 Tg mice and non-Tg littermates (Mann-Whitney U test). As not only simple deletions of mtDNA but also D-loop-containing multimers accumulated in the brains of Tg mice, it is not possible to convert the CO1/D-loop ratio to a percentage of the number of molecules of deleted mtDNA. However, the smaller value of the ratio indicates that brain-derived deletions and multimers of mtDNA are present in the plasma of Tg mice. Bars indicate average values and standard deviations.
Figure 2.

Comparison of the plasma ccf-mtDNA level, ND4/ND1 ratio, and IL-6 levels between remitted state and depressed state of the same patients with bipolar disorder.

Bars indicate average values.

a) Comparison of plasma mtDNA copy numbers between rBD and dBD (Wilcoxon matched-pairs signed rank test).

b) Comparison of plasma ND4/ND1 ratio between rBD and dBD (Wilcoxon matched-pairs signed rank test).

c) Comparison of plasma IL-6 levels between individuals with rBD and dBD (Wilcoxon matched-pairs signed rank test).

Abbreviations: rBD, remitted state of bipolar disorder; dBD, depressed state of bipolar disorder.
**Intra-individual state-dependent comparison of plasma mitochondrial DNA copy number and IL-6 levels in patients with bipolar disorder**

**Supplementary Information**

Yuki Kageyama, Yasuhiko Deguchi, Takaoki Kasahara, Munehide Tani, Kenji Kuroda, Koki Inoue, Tadafumi Kato

Supplementary Figure S1. Correlation between the plasma *ND1* copy number and *ND4* copy number.

Spearman’s rank correlation analysis was performed between two regions of the human plasma ccf-mtDNA (*ND1* and *ND4*).
Supplementary Figure S2. The relationship between the plasma ccf-mtDNA copy number and delusional symptoms in dBD.

Spearman’s rank correlation analysis was performed between the plasma ccf-mtDNA copy number and 21-item Hamilton Depression Rating Scale delusion score in bipolar disorder patients.

Abbreviation: dBD, depressed state of bipolar disorder.
Supplementary Figure S3. Correlation between the plasma ND1 copy number and IL-6 levels in dBD.

Spearman’s rank correlation analysis was performed between the plasma ccf-mtDNA copy number and plasma IL-6 levels in dBD.

Abbreviation: dBD, depressed state of bipolar disorder.
**Supplementary Table S1. Correlation between the plasma mtDNA copy number and medication in rBD and dBD**

<table>
<thead>
<tr>
<th>Medication</th>
<th>n</th>
<th>( \rho )</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lithium carbonate, mg/day (ratio)</td>
<td>9</td>
<td>0.33</td>
<td>0.39(^a)</td>
</tr>
<tr>
<td>Sodium valproate, mg/day (ratio)</td>
<td>14</td>
<td>-0.28</td>
<td>0.32(^a)</td>
</tr>
<tr>
<td>Carbamazepine, mg/day (ratio)</td>
<td>2</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>Lamotrigine, mg/day (ratio)</td>
<td>6</td>
<td>0.0015</td>
<td>0.39(^a)</td>
</tr>
</tbody>
</table>

\(^a\)Spearman’s rank correlation analyses

Abbreviations: dBD, depressed state of bipolar disorder; rBD, remitted state of bipolar disorder; N.A., not applicable.