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ORIGINAL CONTRIBUTION

Mitochondrial DNA deletions of blood lymphocytes as genetic markers of low folate-related mitochondrial genotoxicity in peripheral tissues

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Abstract
Background A low folate status and mitochondrial DNA (mtDNA) mutations are risk factors for various cancers and degenerative diseases. It is not known if lymphocytic mtDNA deletions can be used as genetic "markers" to reflect global mtDNA damage during folate deficiency.
Aim of the study The aim of this study was to characterize folate-related mtDNA deletions in lymphocytes and their associations with mt genotoxicity in peripheral tissues.
Methods Weaning Wistar rats were fed folate-deficient and folate-replete (control) diets for 2 and 4 weeks. Folate levels of blood lymphocytes and various tissues were assayed by the *Lactobacillus casei* method. mtDNA deletions were measured by a real-time polymerase chain reaction analysis of whole DNA extracts.
Results Compared to the control counterparts, mtDNA deletions of lymphocytes increased by 3.5-fold ($P < 0.05$) after 4 weeks of folate deficiency. Lymphocytic mtDNA deletions were inversely associated with plasma ($r = -0.619$, $P = 0.018$), red blood cell ($r = -0.668$, $P = 0.009$), and lymphocytic folate levels ($r = -0.536$, $P = 0.048$). Frequencies of lymphatic mtDNA deletions were positively correlated with mtDNA deletions in tissues including the lungs ($r = 0.803$, $P = 0.0005$), muscles ($r = 0.755$, $P = 0.001$), heart ($r = 0.633$, $P = 0.015$), liver ($r = 0.722$, $P = 0.003$), kidneys ($r = 0.737$, $P = 0.006$), pancreas ($r = 0.666$, $P = 0.009$), and brain ($r = 0.917$, $P < 0.0001$). *Conclusions*: Our data demonstrate that accumulated mtDNA deletions of lymphocytes depended upon dietary folate deprivation. The accumulated mt deletions in lymphocytes closely reflected the mt genotoxicity in the peripheral tissues during folate deficiency.

Keywords Folate deficiency · mtDNA deletions · Lymphocytes · Genetic marker · Peripheral tissues

Abbreviations

FD	Folate-deficient
mt	Mitochondrial
$mtDNA^{4834}$ deletion	4,834-bp large deletion in mtDNA
ROS	Reactive oxygen species
RE	Relative expression

Introduction

Mitochondrial DNA (mtDNA) is vulnerable to oxidative injuries due to the proximity of generated reactive oxygen species (ROS) and a low level of DNA repair [30]. mtDNA damage including mutations and/or rearrangements are considered to play an important role in the pathology of human disease. Single mtDNA deletions consist of an identical and clonally expanded deleted mtDNA molecule present in all cells within an affected tissue including blood cells, which is a common cause of sporadic mt diseases such as mt myopathies [13], Kearns–Sayre syndrome [22], Pearson's syndrome, and progressive external ophthalmoplegia [29].

Multiple mtDNA deletions may accumulate in postmitotic tissues of patients with inherited mutations in nuclear genes such as mtDNA gamma polymerase (POLG1) involved in mtDNA maintenance [27]. Multiple mtDNA deletions were also described in aged tissues and in patients with neurodegenerative disorders [27] and cancers [12].

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Among various types of multiple mtDNA deletions, large-scale deletions of mtDNA—a 4977-bp deletion in humans and a 4834-bp deletion (mtDNA^{4834} deletion) in rodents—were commonly found to accumulate in aging tissues in humans [7, 21, 38] and tumor specimens [12, 17, 43]. The percent of mtDNA deletions in such cases is usually much lower than that seen in patients with mt disorders. As mtDNA deletions accumulate beyond a certain threshold, these mt genetic lesions are associated with pathological and physiological defects including oxidative injuries, respiratory dysfunction, and tissue injuries [39].

Folate insufficiency is thought to influence DNA stability [4, 11]. Folate deprivation leads to both chromosomal DNA injuries [10, 31] and mtDNA mutations [2, 5]. Rats given folic acid supplementation have fewer common mtDNA deletions in hepatic tissues upon chemotherapeutic drug treatment [2] or in aging liver tissues [8]. Four weeks of folate deficiency increased the frequency of large mtDNA deletions in several tissues of young rats [5]. A low folate status or accumulation of mtDNA mutations was associated with mt dysfunction, vicious ROS-generated cycles, and premature cell death [3, 32]. Low folate-associated chromosomal and mt genomic instability was proposed as one of the plausible mechanisms in several diseases such as hepatocellular carcinoma [2, 20] and Alzheimer's disease [24].

To detect disease-related and folate-associated genomic instability at an early time, tissue biopsies are not practical for routine clinical screening. Increased mtDNA deletions are consistently reported in the blood of patients with Kearns–Sayre syndrome, Pearson's pancreas syndrome, and mt myopathies [1, 36]. An assay of mtDNA deletions and folate performed on samples of venous blood may serve as useful markers for monitoring folate-related global mt genotoxicity. However, little is known about the role of lymphocytic mtDNA aberrations in relation to the folate status and global mt genetic impairment. The aim of this study was to characterize mtDNA deletions in lymphocytes during folate deprivation and their association with mt genotoxicity in various tissues. Applying a real-time polymerase chain reaction (PCR) method, we measured mtDNA deletions in lymphocytes and several tissues of rats fed control and folate-deficient (FD) diets for 2 and 4 weeks. Relationships among lymphocytic mtDNA deletions, the folate status, and global mt genotoxicity in peripheral tissues are discussed.

Materials and methods

Animals and experimental diets

An L-amino acid-defined FD diet with 1% succinylsulfathiazole was specially formulated by Harlan Teklad (Madison, WI, USA). The basal FD diet supplemented with 8 mg folic acid/kg was designated the control diet [37]. Male weanling Wistar rats ($n = 18$) were randomly assigned to the FD or control diet using a pair-fed model [5]. After 2- and 4-week feeding periods, rats were sacrificed to collect blood and tissues. Tissues were quickly excised, immediately frozen in liquid nitrogen, and stored in a −80 °C freezer until analysis. Ficoll gradient-purified blood cells designated the lymphocyte fraction were cryopreserved at −80 °C. The experimental protocols were approved by the Institutional Animal Care Committee of Fu-Jen University.

Blood, tissue, and intracellular compartmental folate assay

Samples for the folate analysis were prepared according to Varela-Moreiras and Selhub [34]. After incubation of the thawed sample extracts with chicken pancreas conjugase (v/v 4:1) at 37 °C for 6 h, a microbiologic assay was performed using cryoprotected *Lactobacillus casei* in 96-well microtiter plates [14].

Quantification of mtDNA^{4834} deletion and contents

The amount of mtDNA^{4834} deleted was determined by co-amplifying the mtDNA D-loop versus the deleted gene using a real-time PCR assay. Primers for the mtDNA D-loop and mtDNA^{4834} deletion were previously described elsewhere [5]. PCR amplification was carried out in a 50-μl reaction consisting of 1× TaqMan Universal Master Mix, 200 nM of each mtDNA^{4834} deletion primer, 100 nM of each D-loop primer, and 100 nM of each mtDNA^{4834} deletion and D-loop probe primer. The cycling conditions included an initial phase of 2 min at 50 °C and 10 min at 95 °C, then 40 cycles of 15 s at 95 °C and 0.5 min at 72 °C. The fluorescence spectra were monitored by the LightCycler Detection System (LightCycler, Roche Diagnostics, Mannheim, Germany). The cycle at which a statistically significant increase in normalized fluorescence was first detected was designated the threshold cycle number (C_t). The relative mtDNA^{4834} deletion/mtDNA was expressed by the ΔC_t mt deletion (mt $C_{t\ del}$ − mt $C_{t\ D\text{-}loop}$), given the fact that the efficiencies of the PCR reaction for detecting both the mtDNA^{4834} deletion and mtDNA D-loop region were similar [5]. A smaller ΔC_t deletion indicates more deletions. The relative expression (RE) indicates multiples of difference in deletions of the 2- and 4-week FD groups with the respective control group. The RE was calculated using the equation $2^{-\Delta\Delta C_t}$ where $\Delta\Delta C_t = \Delta C_{t\ FD\ mtDNA\ deletion} - \Delta C_{t\ control\ mtDNA\ deletion}$. The relative amount of mtDNA content relative to nuclear genomic DNA was determined by co-amplifying the mitochondrial

D-loop versus the β-actin gene using a real-time PCR assay. Details of the protocol were previously described elsewhere [5].

Statistical methods

Data are presented as the mean ± SD. The effects of dietary folate deprivation on the folate status, mtDNA deletions, and relative mtDNA contents were analyzed by one-way analysis of variance and Duncan's multiple-range test using the general linear model of SAS Institute (Cary, NC, USA). Differences were considered significant at $P < 0.05$. Pearson's correlation coefficients were used to measure the association between lymphocytes and tissue mtDNA damage.

Results

Intracellular and compartmental folate levels of lymphocytes and tissues in rats fed the control and FD diets

Compared to values of the control counterparts, lymphocytic folate levels of rats fed the FD diet for 4 weeks had decreased by 43% (Table 1). In five measured tissues of rats, intracellular folate levels were significantly reduced after 2 weeks of the FD diet. Among the measured tissues, lungs of rats had the most-reduced folate levels (35% of the controls) compared to the other tissues. With the exception of the lungs, tissues folate levels of rats displayed further drops after feeding the FD diet for an additional 2 weeks (4 weeks total; $P < 0.05$). Figure 1 shows the folate status in the cytosolic and mt pools of lymphocytes, and tissues of rats fed the control and FD diets. mt Folate levels of lymphocytes had significantly decreased in the initial 2 weeks of folate deficiency, whereas changes of cytosolic folate in lymphocytes responded to 4 weeks of folate deficiency ($P < 0.05$). Cytosolic and mt folate levels of tissues decreased in rats fed the FD diets for 2 and 4 weeks.

mtDNA alterations in lymphocytes and various tissues of rats fed the control and FD diet

By real-time PCR analysis, spontaneous or background "mtDNA deletions" were present in lymphocytes and various tissues of rats fed the control diet (Table 2). Compared to the controls, 2 weeks of the FD diet led to a 2.5-fold increase in the frequency of mtDNA deletions in rat lymphocytes ($P < 0.05$). After consuming the FD diet for 4 weeks, lymphocytic mtDNA deletions of rats had further accumulated to 3.5-fold the control value. Frequencies of mtDNA deletions in the spleen, muscles, and stomach had significantly increased by 3.1-, 2.4-, and 1.9-fold, respectively, compared to the control counterparts ($P < 0.05$). Increased frequencies of mtDNA deletions were also found in the lungs (1.4-fold) and small intestines (1.2-fold) of rats fed the FD diet for 4 weeks. Compared to control values, 2 weeks of the FD diet did not significantly alter mtDNA contents of any measured tissues of rats (data not shown). Relative mtDNA copy numbers of lymphocyte and muscles of rats fed the FD diet for 4 weeks significantly increased with RE values of 1.93 and 2.28, respectively.

Correlations between lymphocytic mtDNA mutations and the blood folate status

Relationships between lymphocytic mtDNA deletions (the present study) and blood folate levels of rats fed the control and FD diets (data adapted from ref. [5]) were

Table 1 Folate levels in lymphocytes and various tissues of rats fed the control and folate-deficient (FD) diets

Lymphocytes and tissues	Control	2 Weeks of FD	4 Weeks of FD
nmol/mg Protein (% of control)			
Lymphocytes	0.910 ± 0.088^{a} (100)	0.914 ± 0.061^{a} (100)	0.517 ± 0.046^{b} (57)
nmol/g Tissue (% of control)			
Spleen	8.770 ± 0.109^{a} (100)	4.922 ± 0.228^{b} (56)	4.029 ± 0.418^{c} (46)
Small intestines	8.083 ± 0.608^{a} (100)	5.196 ± 0.659^{b} (64)	3.402 ± 0.425^{c} (42)
Lungs	6.807 ± 0.667^{a} (100)	2.366 ± 0.258^{b} (35)	2.392 ± 0.487^{b} (35)
Stomach	6.417 ± 0.254^{a} (100)	3.910 ± 0.305^{b} (61)	3.486 ± 0.418^{c} (54)
Muscles	5.220 ± 0.459^{a} (100)	3.032 ± 0.153^{b} (58)	2.355 ± 0.433^{c} (45)

Weaning Wistar male rats were fed either the folate-replete (control) or FD diet for 2 and 4 weeks. Lymphocytes and various tissues were isolated from the control and FD rats. Folate levels were analyzed by the *L. casei* assay as described in "Materials and methods". Data are the mean ± SD (n = 6 for each group). Statistical differences were determined by one-way ANOVA followed Dunnett's multiple-range test

Values in the same row with different superscripts significantly differ at $P < 0.05$

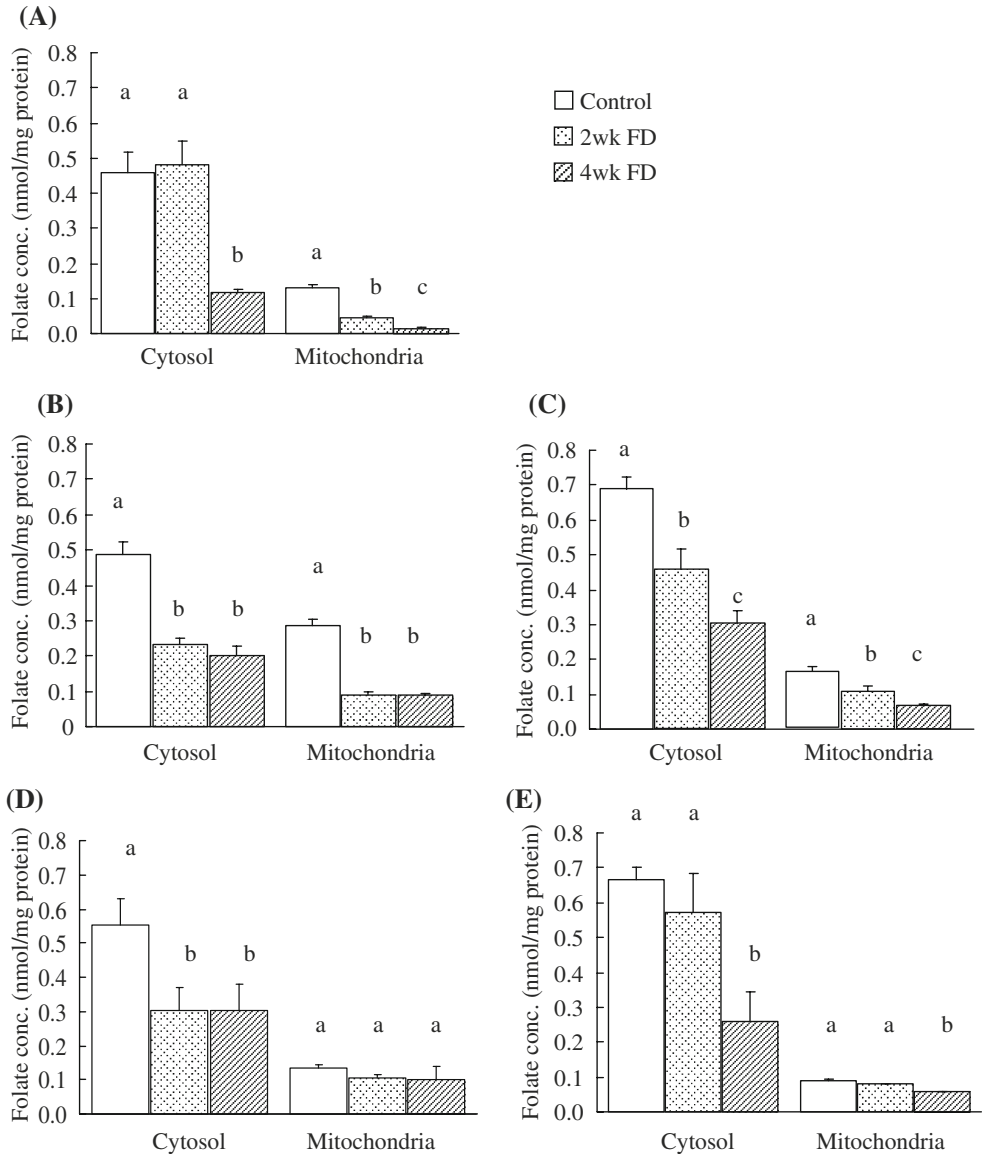

Fig. 1 Compartmental folate status of lymphocytes and peripheral tissues of rats fed the control and folate-deficient (*FD*) diets. **a** Lymphocytes, **b** lung, **c** stomach, **d** muscle, and **e** small intestine. Values are the mean ± SD ($n = 6$ for each group). Statistical differences were determined by one-way ANOVA followed by Dunnett's multiple-range test. Values with different letters significantly differ at $P < 0.05$

assayed. Figure 2 shows that lymphocytic mtDNA deletions were significantly correlated with levels of RBC folate ($r = -0.668$, $P = 0.009$) and plasma folate ($r = -0.619$, $P = 0.018$).

Associations among the lymphocytic folate status, common mtDNA deletions, and tissue mtDNA deletions

Using pooled mtDNA deletion data from ten tissues of control and FD animals (data from the present study and from ref. [5]), relationships among lymphocytic mtDNA deletions, tissue mtDNA mutations, and the lymphocytic folate status are presented in Table 3. Increased mtDNA deletions in lymphocytes were associated with accumulated mtDNA deletions in the brain ($r = 0.917$, $P < 0.0001$), lungs ($r = 0.803$, $P = 0.0005$), muscles ($r = 0.756$, $P = 0.001$), kidneys ($r = 0.737$, $P = 0.006$), liver ($r = 0.722$, $P = 0.003$), pancreas ($r = 0.666$, $P = 0.009$), and heart ($r = 0.634$, $P = 0.015$). Folate levels in lymphocytes were associated with mtDNA mutations in the heart ($r = -0.544$, $P = 0.019$), liver ($r = -0.761$, $P = 0.0002$), kidneys ($r = -0.745$, $P = 0.002$), pancreas ($r = -0.820$, $P < 0.0001$), brain ($r = -0.725$, $P = 0.001$), and lungs ($r = -0.501$, $P = 0.045$).

Table 2 Changes in the accumulation of mitochondrial DNA (mtDNA) deletions in various tissues of rats fed the control or folate-deficient (FD) diet for 2 and 4 weeks

Lymphocytes and various tissues	Controls		2 Weeks of FD		4 Weeks of FD	
	ΔC_t[a]	RE[b]	ΔC_t	RE	ΔC_t	RE
Lymphocytes	4.25 ± 0.59[a]	1	2.92 ± 0.95[b]	2.5	2.41 ± 0.77[b]	3.5
Spleen	4.75 ± 1.28[a]	1	3.77 ± 0.52[ab]	1.9	3.11 ± 0.76[b]	3.1
Muscles	4.41 ± 0.82[a]	1	3.99 ± 0.40[a]	1.3	3.11 ± 0.26[b]	2.4
Stomach	4.15 ± 0.52[a]	1	3.77 ± 0.64[ab]	1.3	3.20 ± 0.44[b]	1.9
Lungs	3.94 ± 1.71[a]	1	3.73 ± 0.16[a]	1.1	3.38 ± 0.53[a]	1.4
Small intestines	3.86 ± 1.27[a]	1	3.71 ± 0.82[a]	1.1	3.59 ± 0.81[a]	1.2

Weaning Wistar rats were fed the FD or folate-replete (control) diet for 2 or 4 weeks. mtDNA^{4834} deletions were analyzed by real-time PCR as described in "Materials and methods". Values are the mean ± SD ($n = 6$ in each group). Statistical differences were determined by one-way ANOVA followed by Duncan's multiple-range test

Values in the same row with different superscripts significantly differ at $P < 0.05$

[a] The ratio of mtDNA^{4834} deletions to mtDNA was calculated using $\Delta C_t = \text{mt } C_{t\ \text{del}} - \text{mt } C_{t\ \text{D-loop}}$

[b] The relative expression (RE) indicates the factorial difference in deletions between 2 weeks of the FD diet, 4 weeks of the FD diet, and the controls. For details of the calculations, see "Materials and methods"

Discussion

Although several researchers detected large deletions of human mtDNA in blood cells [26, 33, 36], very little is known about modification of mtDNA deletions in lymphocytes associated with nutritional factors. Our data, for the first time, reveal a folate-dependent accumulation of mtDNA deletions in lymphocytes. By applying a real-time PCR method, frequencies of mtDNA deletions in lymphocytes of rodents were found to depend upon changes in serum, RBC, and lymphocyte folate levels. Accumulated mtDNA deletions in lymphocytes initially being detected at the early stage of 2 weeks of folate depletion (a 2.5-fold increase) and continuing to be detectable after 4 weeks of folate deficiency suggest that accumulated lymphocytic mtDNA deletions may serve as a biomarker of mt genomic instability responding to dietary folate deprivation as well as depleted cellular folate storage.

Mechanisms of low folate-dependent accumulation of mtDNA deletions in lymphocytes are not clearly understood. The accumulated mtDNA deletions in FD lymphocytes could be a direct effect of reduced compartmental folate levels, in particular of lower mt folate concentrations (Fig. 1). It was demonstrated that accumulated mtDNA deletions depend upon mt folate levels [5]. Decreased mt folate causes mt oxidative decay as a result of impaired mt antioxidant enzymatic activities, increased mt oxidative protein injuries, respiratory dysfunction, and ROS overproduction [3]. It was reported that increased oxidative stress is commonly associated with accumulation of mtDNA deletions in several aging tissues [40–42]. Consistently, altered antioxidant defenses and DNA repair mechanisms were shown to affect levels of mtDNA damage in lymphocytes [9, 25].

Although damaged mtDNA might not accumulate in any significant extent in normal human lymphocytes due to the high turnover rate of blood cells and active turnover of mutated mtDNA in mitotic cells [26, 28], lymphocytic mtDNA deletions continued to accumulate by 3.5-fold after 4 weeks of folate deficiency (Table 2). This persistent rise in lymphocytic mtDNA deletions during the prolonged 4 weeks of folate deficiency may have resulted from a secondary effect of induced mt biogenesis, a biological strategy to rescue mitochondria with genetic lesions. In order to compensate for oxidative stress-related dysfunctional mitochondria, oxidative injuries usually trigger mt biogenesis of mammalian cells by upregulation of mt biogenesis nuclear factors (mt transcriptional factor A and nuclear respiratory factor 1) to increase mtDNA replication [16, 35]. The fact that the mtDNA copy number of 4-week FD lymphocytes had increased by 1.93-fold following the accumulation of lymphocytic mtDNA deletions supports the plausibility of this mechanism. Taken together, elevated oxidative stress due to reduced compartmental folate levels and the triggering of mtDNA biogenesis associated with oxidative injuries may be partially, if not totally, attributable to accumulated mtDNA deletions in FD lymphocytes.

It should be noted that the percentage of mtDNA deletions observed in lymphocytes seems too low to cause any significant biochemical defects. mtDNA deletions are usually recessive, so that a minimum of 60% deletions is required to demonstrate a mt defect [7, 13]. In spite of the possibly undetectable mt defects in lymphocytes, one of our major findings is that accumulated mt deletions in lymphocytes closely reflected mt genotoxicity in peripheral tissues (Table 3). During dietary folate deprivation, the accumulation of mtDNA deletions was simultaneously

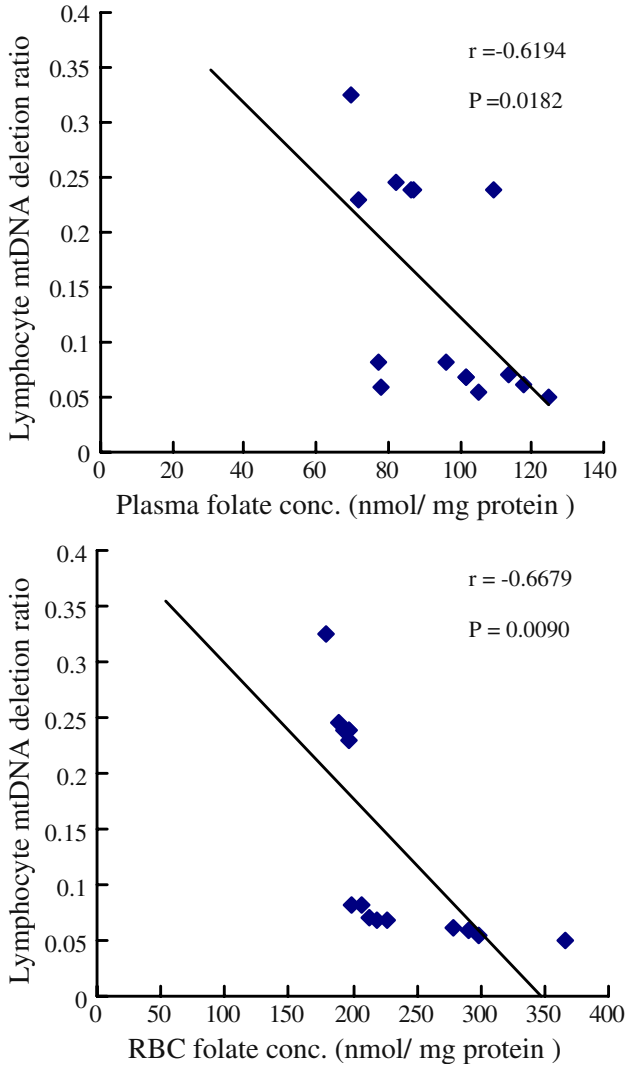

Fig. 2 Pearson's correlation coefficients for relationships among expression of lymphocytic $\Delta mtDNA^{4.8\ kb}$, red blood cell (*RBC*) folate, and plasma folate levels of rats fed diets with different folate concentrations for 2 or 4 weeks

observed in postmitotic tissues like the brain, liver, heart, kidneys, pancreas [5], and muscles (Table 2). This low folate-associated distribution of altered mtDNA deletions in FD tissues of young rats resembled the age-related pattern of mtDNA damage which accumulates in aging human tissues such as the brain [6], heart [18], liver [21], and skeletal muscles [38]. Like mtDNA damage which accumulated in FD lymphocytes, both mitotic and post-mitotic tissues of mammals during folate deficiency encounter oxidative stress as a result of reduced intracellular folate levels [5] and elevated homocysteine levels, a strong prooxidant which can cause oxidative injuries to tissues [23]. Folate deprivation and elevated homocysteine blood levels are associated with an impaired antioxidant capability, increased oxidative injuries [15], and more

Table 3 Pearson correlation coefficients for relationships among frequencies of large mtDNA deletions in lymphocytes and peripheral tissues, and the lymphocytic folate status

$\Delta mtDNA^{4.8\ kb}$ in various tissues	Lymphocyte $\Delta mtDNA^{4.8\ kb}$		Lymphocyte folate levels	
	r	*P* value	*r*	*P* value
Lungs	0.8036	0.0005	−0.5006	0.0452
Muscles	0.7558	0.0011	−0.4993	0.0691
Spleen	0.3400	0.2343	−0.5802	0.0146
Stomach	−0.1823	0.5511	−0.3659	0.1982
Small intestines	0.0464	0.8695	−0.3462	0.1594
Heart	0.6336	0.0150	−0.5439	0.0196
Liver	0.7223	0.0035	−0.7607	0.0002
Kidneys	0.7374	0.0062	−0.7453	0.0022
Pancreas	0.6666	0.0092	−0.8204	<0.0001
Brain	0.9174	<0.0001	−0.7252	0.0015
Lymphocytes	–	–	−0.5364	0.0480

oxidative 8-OHdG lesions in rat livers [5]. Thus, folate deprivation may challenge global organs of mammals including lymphocytes as well as peripheral tissues with elevated oxidative stress and consequently induce generalized mt genotoxicity in whole organisms [3, 5, 15, and the present study]. In this regard, the lymphocytic folate status and mtDNA damage might therefore reflect global mtDNA genotoxicity in peripheral tissues. It should be noted that mtDNA injuries of a few tissues such as the small intestines were not correlated with altered mtDNA deletions or reduced folate levels of lymphocytes during folate deficiency. mtDNA may be actively turned over in the small intestines as enterocytes rapidly divide every 1 ~ 3 days, therefore ridding these cells of many of the mutant forms [19].

In summary, our data demonstrate that accumulated lymphocytic mtDNA deletions depended upon dietary folate deprivation as well as depletion of cellular folate storage. mtDNA deletions in FD lymphocytes can possibly be used as natural genetic "markers" to mirror the global mtDNA genotoxicity of disease-susceptible tissues during folate deficiency. Further human studies are warranted to verify the mtDNA mutations of blood lymphocytes as a biomarker for mt toxicity in low folate-associated and clinically affected tissues. Studies on the minimum daily dose of folate able to prevent mtDNA deletions in lymphocytes and peripheral tissues are warranted.

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