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Oxidative Stress-related Alteration of the Copy Number of Mitochondrial DNA in Human Leukocytes

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The role of oxidative stress in the regulation of the copy number of mitochondrial DNA (mtDNA) in human leukocytes is unclear. In this study, we investigated the redox factors in plasma that may contribute to the alteration of mtDNA copy number in human leukocytes. A total of 156 healthy subjects of 25–80 years of age who exhibited no significant difference in the distribution of subpopulations of leukocytes in blood were recruited. Small-molecular-weight antioxidants and thiobarbituric acid reactive substances (TBARS) in plasma and 8-hydroxy-2′-deoxyguanosine (8-OHdG) and 4,977 bp deletion of mtDNA in leukocytes were determined. The mtDNA copy number in leukocytes was determined by real-time PCR. The results showed that the copy number of mtDNA in leukocytes was changed with age in a biphasic manner that fits in a positively quadratic regression model ($P = 0.001$). Retinol ($P = 0.005$), non-protein thiols ($P = 0.001$) and ferritin ($P = 0.004$) in plasma and total glutathione in erythrocytes ($P = 0.046$) were the significant redox factors that correlated with the mtDNA copy number in leukocytes in a positive manner. By contrast, α-tocopherol levels in plasma ($P = 0.001$) and erythrocytes ($P = 0.033$) were negatively correlated with the mtDNA copy number in leukocytes. Three oxidative indices including the incidence of 4,977 bp deletion of mtDNA ($P = 0.016$) and 8-OHdG content in leukocytes ($P = 0.003$) and TBARS in plasma ($P = 0.001$) were all positively correlated with the copy number of mtDNA in leukocytes. Taken these findings together, we suggest that the copy number of mtDNA in leukocytes is affected by oxidative stress in blood circulation elicited by the alteration of plasma antioxidants/prooxidants and oxidative damage to DNA.

Keywords: Mitochondrial DNA; 8-Hydroxy-2′-deoxyguanosine; Copy number; Deletion; Lipid peroxide; Aging

INTRODUCTION

Mitochondria are intracellular organelles that provide most of the energy consumed by human cells. High rate of respiration and oxidative phosphorylation in the brain, heart and skeletal muscle is related to their high demand for energy. Thus, the number of mitochondria should be adjusted according to the energy demand of tissue cells.[1] Alteration of the copy number of mitochondrial DNA (mtDNA) or mitochondrial mass in affected tissues has been considered as one of the factors involved in the pathogenesis of oxidative phosphorylation disorders.[2–7] Thus, the levels of antioxidants and prooxidants may play a role in the mechanism of adjusting mitochondrial mass or mtDNA copy number in affected tissue cells.

Human leukocyte is a good target cell for the study of the relationship between alteration of mtDNA copy number and change of antioxidants and prooxidants status not only due to easy sampling but also the characteristics of high demand of aerobic metabolism during immune response. Moreover, mitochondrial respiratory function and antioxidant capability of immuno-competent cells have been thought as two factors determining the ability of leukocytes to cope with the oxidative stress in human immunosenescence.[8] In the present study, only the leukocytes with normal range of subpopulations were recruited to prevent the bias of distinct response

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of mtDNA replication in different cell type of leukocytes. Plasma levels of urate, albumin, ascorbate, α-tocopherol, retinol, protein thiols, non-protein thiols, homocysteine and ferritin as well as RBC levels of α-tocopherol and total glutathione were determined for the assessment of the redox status in blood circulation. In addition, three indices were measured including thiobarbituric acid reactive substances (TBARS) in plasma, 4,977 bp deletion of mtDNA and 8-hydroxy-2′-deoxyguanosine (8-OHdG) in leukocytes, which are good indicators of oxidative damage to plasma lipids and damage to mtDNA and nuclear DNA in leukocytes, respectively.[9–12] To the best of our knowledge, this is the first survey to investigate the relationship between the alteration of mtDNA copy number in leukocytes and oxidative stress in plasma as well as DNA damage in leukocytes.

MATERIALS AND METHODS

Subjects

Two hundred healthy subjects were recruited from the health clinic of Changhua Christian Hospital, Taiwan, from September 2001 to October 2002. Informed consent for study enrollment was obtained from each of the subjects. All of the human experimental procedures followed the ethical guideline of Changhua Christian Hospital. General physical examination and blood biochemistry studies had been performed to exclude those with a systemic disease such as hypertension, diabetes or hyperlipidemia. All enrolled subjects showed a normal distribution in leukocyte subpopulations and a total of 156 healthy subjects were finally recruited in this study. The demographic characteristics, leukocyte subpopulations and blood biochemistry data are summarized in Table I. Blood samples were drawn from each subject in the morning after overnight fasting. For each subject, 20 ml of whole blood was withdrawn from an antecubital vein and quickly delivered into an ethylenediamine tetraaceticacid (EDTA)-containing plastic tube. Blood plasma was collected by centrifugation of blood at 300*g* for 10 min, divided into several aliquots, and stored in liquid nitrogen until analysis, except for the samples that were immediately used for measurement of plasma levels of non-protein thiols and ascorbate. Erythrocytes were prepared by centrifugation of fresh blood and washing for three times of the packed cells with phosphate-buffered saline (PBS, 8.9 mM Na_2HPO_4, 1.1 mM NaH_2PO_4, 140 mM NaCl, pH 7.4). The washed erythrocytes were suspended in PBS containing 10 mM glucose with a hematocrit value of 10%, and subjected to the measurement of total glutathione (GSH) and α-tocopherol. Washed leukocytes were quickly separated from plasma as the buffy coat layer and immediately subjected to DNA isolation.[13]

TABLE I Characteristics of the subjects recruited in this study ($n = 156$)

	Mean ± SD
Demographic data	
Age (year)	57 ± 15
Sex (male/female)	86/70
BMI (kg/m^2)	24 ± 3
Smoking (smoker/non-smoker)	53/103
Leukocyte (10^3/μl)	6.88 ± 2.46
Neutrophils (%)	61 ± 13*
Lymphocytes (%)	30 ± 7*
Monocytes (%)	6 ± 3*
Eosinophils (%)	1 ± 5*
Basophils (%)	0 ± 2*
Blood biochemistry	
Glucose (mg/dl)	101 ± 13
Homocysteine (μM)	9.57 ± 2.30
Ferritin (ng/ml)	163.1 ± 49.7
Urate (mg/dl)	5.9 ± 1.7
Albumin (g/dl)	4.04 ± 0.29
Ascorbate (μM)	69.3 ± 27.5
α-Tocopherol (μM)	13.4 ± 9.3
Erythrocyte α-tocopherol (μM)	4.1 ± 2.2
Retinol (μM)	1.57 ± 0.45
Protein thiols (μM)	332.9 ± 73.23
Non-protein thiols (μM)	32.51 ± 10.54
Erythrocyte GSH (nmol/mg protein)	8.3 ± 4.9
MtDNA copy number/nuclear gene	1375 ± 2492
8-OHdG /10^{6}dG	23 ± 34
TBARS (nM)	3.2 ± 2.1

BMI, body mass index; MtDNA, mitochondrial DNA; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; TBARS, thiobarbituric acid reactive substances. *$P > 0.05$ by the Levene test of homogeneity of variance.

Isolation of Leukocyte DNA

Total cellular DNA of leukocytes was extracted by phenol/chloroform after lysis with proteinase K in an alkaline SDS solution as described previously.[14] The leukocytes were first washed with ice-cold PBS (pH 7.3) containing 0.1 mM deferoxamine mesylate (DFAM), and were then lysed in 1.5 ml of the TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) that contained 2 mM butylated hydroxyltoluene (BHT), 100 μg/ml RNase A and 0.5% SDS. The cell lysate was further incubated at 37°C for 1 h. Proteinase K was added to a final concentration of 100 μg/ml and the mixture was then incubated at 55°C overnight. After addition of NaCl to 150 mM, the lysate was extracted each with an equal volume of phenol, phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1), respectively. DNA was precipitated by the addition of 1/10 volume of 3 M sodium acetate (pH 5.2) and 2.5 volume of ice-cold ethanol. Finally, the DNA was collected by centrifugation at 10,000*g* for 15 min at 4°C, washed with 70% ethanol (v/v), and dried by flushing with argon gas to prevent oxidative damage.

Determination of mtDNA Copy Number in Human Leukocytes

The fluorescence-based quantitative PCR (QPCR) was used to determine the mtDNA copy number in human leukocytes according to the method developed by Wong *et al.*[15] with some modifications. Essentially, it was carried out by using the LightCycle™-FastStart DNA Master SYBR Green I kit supplied by Roche Molecular Biochemicals (Pleasanton, CA). Two primers (forward: 5′-GAAGAGCCAAGGAC AGGTAC-3′; reverse: 5′-CAACTTCATCCACGTTCACC-3′) complementary to the sequences of the β-globin gene were used to amplify a 268-bp product. QPCR was performed under the following conditions: initial denaturation at 95°C for 300 s followed by 40 cycles of 0.1 s at 95°C, 6 s at 58°C and 18 s at 72°C. For studying the mitochondrial gene products, the forward primer 5′-AACATACCCATGGCCAACCT-3′ and the reverse primer 5′-AGCGAAGGGTTGTAGTAGCCC-3′, which are complementary to the sequence of the mitochondrial ND1 gene, were used to amplify a 153-bp PCR product. QPCR was performed under identical conditions except that extension time was 8 s. A total of 20 ng DNA was used in QPCR for determination of the threshold cycle number (Ct) of the nuclear and mitochondrial genes, respectively. The Ct values for β-globin gene and mitochondrial ND1 gene were concurrently determined in each QPCR run. The efficiency of QPCR and standard regression analyses were performed during each run with different amounts of two standard DNA fragments. One was a 268-bp DNA fragment of β-globin gene, which was amplified from 1.2×10^{-5} to 1.2×10^{1} ng of leukocyte DNA using the primers of 5′-GAAGAGCCAAGGACAGGTAC-3′ and 5′-CAACTTCATCCACG TTCACC-3′. The other was a 153-bp DNA fragment of the ND1 gene amplified from 1.2×10^{-4} to 1.2×10^{2} ng DNA by using the primers of 5′-GATGGTGCAGCCG CTATTAA-3′ and 5′-AGGTTGGCCATGGGTATGTT-3′ (Fig. 1). The Ct values were accepted in each QPCR run when the correlation coefficient was greater than 0.98 and when the efficiency was between 1.95 and 2.00. Under such conditions, Ct values could be used to measure the copy number of mtDNA by standard regression analyses.

Detection of 4,977 bp Deletion of mtDNA

The 4,977 bp deletion of mtDNA was detected in human leukocytes by a PCR method.[16] By using the forward primer 5′-GCCCGTATTTACCCTATAGC-3′ and the reserve primer 5′-GGGGAAGCGAGGTTGACCTG-3′), we amplified a 423-bp DNA fragment from mtDNA with 4,977 bp deletion. Each PCR run was done in a 50 μl reaction mixture containing 100 ng of template DNA, 200 μM dNTPs, 1 unit of *Taq* DNA polymerase (Perkin-Elmer/Cetus), 20 pmol of each primer, 50 mM KCl, 1.5 mM $MgCl_2$, and 10 mM Tris–HCl (pH 8.3). A total of 35 cycles of PCR was performed for each sample in a Perkin-Elmer/Cetus DNA thermal cycler. The first cycle consisted of 3 min denaturation at 94°C, 3 min annealing at 55°C and 1 min primer extension at 72°C. The rest of the PCR cycles were then conducted by denaturation at 94°C for 40 s, annealing at 55°C for 40 s and extension at 72°C for 50 s. Direct sequencing of the PCR products was performed on an automated DNA sequencer (ABI Prism 310 DNA Analyzer, PE Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

Measurement of 8-OHdG Content in Leukocyte DNA

The 8-OHdG content in leukocytes was measured by using a high-performance liquid chromatography (HPLC) system equipped with a UV and an electrochemical detectors installed in series (Bioanalytical Systems, West Lafayette, IN) as previously described.[17] Deoxyguanosine (dG; Sigma Chemical Co., St Louis, MO) and 8-OHdG (Cayman, Ann Arbor, MI) were used as standards. By means of repeated analyses ($n = 20$), we found that the intra-assay coefficients of variance (CV) ranged from 5 to 10%, and inter-assay CV ranged from 6 to 14% in the determination of the 8-OHdG content in leukocytes. The 8-OHdG content in total cellular DNA of leukocytes is expressed as 8-OHdG/10^6dG.

Measurement of Plasma Homocysteine

Total homocysteine concentration in plasma was determined as described by Dudman *et al.*[18] An internal recovery standard of *N*-acetylcysteine (1.1 mM, 20 μl) was added to 200 μl plasma to determine the total homocysteine. Plasma sample was treated with tri-*n*-butylphosphine (20 μl, 100 g/l in dimethylformamide) and incubated at 4°C for 60 min before precipitation with 10% trichloroacetic acid containing 1 mM EDTA. Precipitated proteins were removed by centrifugation and 100 μl of the supernatant was incubated with 250 μl of 0.125 M borate (pH 9.2) containing 4 mM EDTA, 20 μl 1.5 N NaOH and 100 μl of SBD-F solution (1 g/l solution in borate/EDTA buffer) for 60 min at 60°C. The solution was cooled to the room temperature and subjected to analysis by HPLC. All derived samples were filtered through a piece of nylon membrane (13 mm × 45 mm, Lita) before injection to the HPLC system (Hitachi Co., Tokyo, Japan). The separation column was a 3.9 mm × 30 cm stainless steel packed with μBondapak C_{18}. A 3 mm × 22 mm guard column, which had been packed with μBondapak C_{18}, was attached to

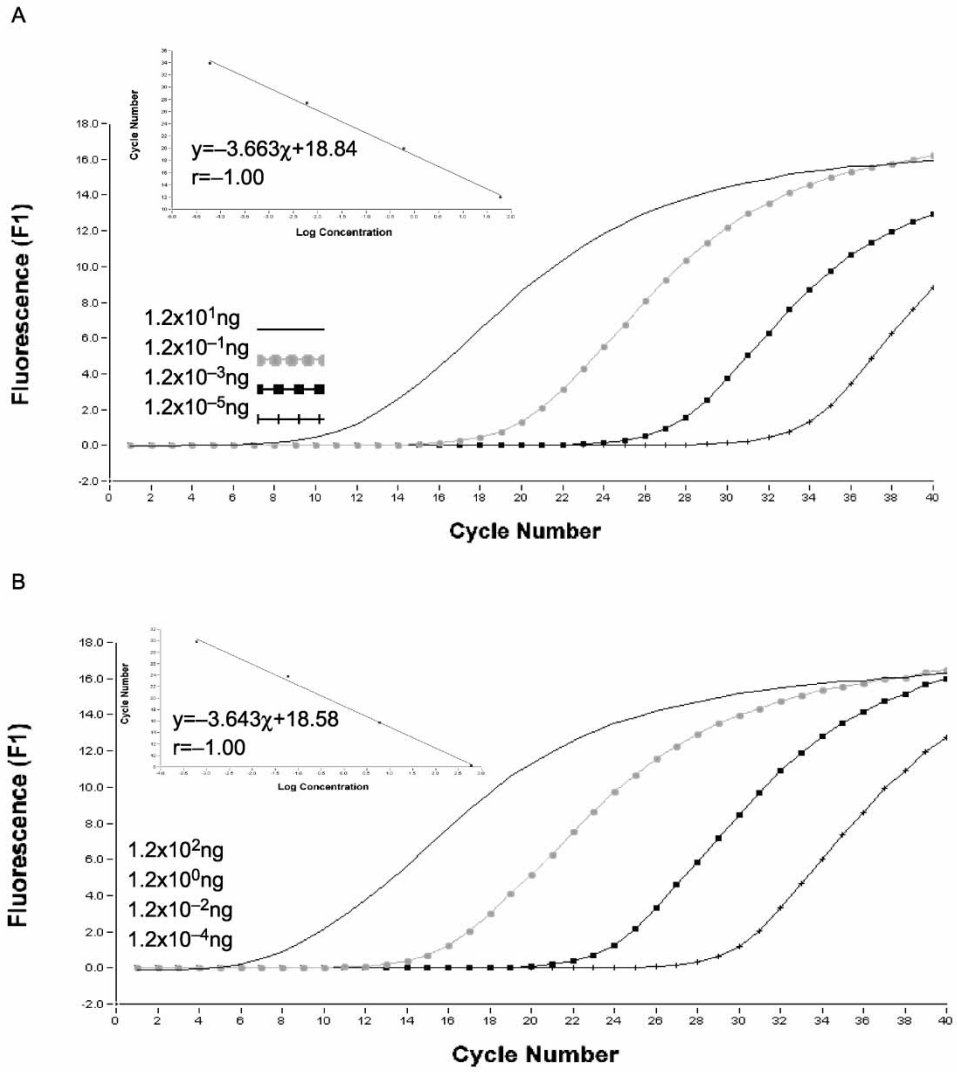

FIGURE 1 Amplification and standard curves for quantitative determination of nuclear and mitochondrial genes. Amplification curves of (A) nuclear β-globin gene and (B) ND1 gene amplified from 1.2×10^{-5} to 1.2×10^{1} ng DNA fragment of β-globin gene or 1.2×10^{-4} to 1.2×10^{2} ng DNA fragment of ND1 gene. The standard curves obtained from the PCR amplifications are embedded in the graphs. The mathematical equations describing the standard curves and correlation coefficients are also shown.

the separation column. The model 7480 fluorescence detector was set at 385 nm for excitation and at 515 nm for emission. With an L-7100 pump, isocratic elution was carried out at a flow rate of 1.2 ml/min with 0.1 M potassium phosphate buffer containing 35 ml/l CH_3CN (pH 3.5). A 50-μl aliquot of each sample was automatically loaded with an L-7200 autosampler. The concentrations of homocysteine in plasma are expressed in μM.

Measurement of Plasma TBARS

Plasma TBARS was measured by using a method modified from the protocol described by Dousseet *et al.*[19] An aliquot of 20 μ1 of plasma was mixed with 2 ml of 0.1 N HCl, 0.3 ml of 10% phosphotungstic acid and 1 ml of 0.7% thiobarbituric acid (TBA). The mixture was heated for 60 min in boiling water, followed by extraction of the TBA adduct with 5 ml of 1-butanol. After centrifugation at 800*g* for 10 min, the fluorescence intensity of the TBA adduct in the 1-butanol layer was quickly measured on a spectrofluorometer set at excitation wavelength of 525 nm and emission wavelength of 555 nm. A calibration curve was constructed for each run by using 1,1,3,3-tetraethoxypropane as standard. The analysis was performed in 1-week after the blood had been withdrawn from the study subject. The concentration of TBARS in plasma is expressed in the unit of μM.

Determination of Plasma Protein Thiols and Non-protein Thiols

The levels of protein thiols and non-protein thiols were determined as described by Boyne and Ellman.[20] An equal volume of 10% perchloric

acid (PCA) was added to 0.05 ml plasma to precipitate proteins, followed by centrifugation at 8000*g* for 10 min. The pellet and supernatant were collected for the determination of protein thiols and non-protein thiols, respectively. The pellet and supernatant were gently suspended in 0.05 ml of 100 mM 5,5′-dithiolbis-2-nitrobenzoic acid and left standing at room temperature for 30 min. After recording the absorbance of the solution, 5 mg of *N*-ethylmaleimide (NEM) was then added to the sample. The NEM has no detectable absorbance at 412 nm, but it decolorizes the reaction product in the assay mixture within 3 min. This permitted us to measure the background absorbance or turbidity, which was then subtracted from the absorbance of each sample solution. The plasma concentrations of protein thiols and non-protein thiols are expressed in μM.

Determination of Total Glutathione in Erythrocytes

The washed erythrocytes were resuspended to a 10% hematocrit value in PBS, containing 100 mM IAA, and was left standing in the dark for 5 min. The cell suspension was then mixed with an equal volume of a solution containing 2.5 mM phenanthroline in 10% PCA to precipitate proteins. After centrifugation, total free GSH was determined by HPLC as described by Reed *et al.*[21] Briefly, the acid-precipitated pellet was washed twice with PCA and then resuspended in 1 ml ether by sonication. After centrifugation at 10,000*g* for 5 min, the resultant pellet was resuspended in 50 mM morpholine-propanesulfonic acid (MOPS) solution (pH 8.5), containing 20 mM dithiothreitol. After incubation for 60 min at 37°C, proteins were again precipitated with 10% PCA and removed by centrifugation. Released GSH in acid solution was measured by the HPLC method. Protein concentrations were determined based on the method of Lowry *et al.*[22] The concentration of total GSH of erytherocytes is expressed in nmol/mg protein.

Determination of Plasma Urate, Albumin and Ferritin Levels

A commercial kit (Sigma Chemical Co., St Louis, MO) was used to determine the concentration of urate in plasma. Briefly, uricase catalyzes the oxidation of uric acid to generate allantoin, CO_2 and H_2O_2. In the presence of peroxidase, H_2O_2 reacts with 4-amino-antipyrine and 3,5-dichloro-2-hydroxybenzenesulfonate to form a quinoneimine dye with an absorbance maximum at 520 nm. The concentration of urate in plasma is expressed in mg/dl. Plasma concentration of albumin was determined by using a Nephelometer BNA (Behringwerke, Marburg, Germany) and is expressed in g/dl. Plasma ferritin level was determined on a Bayer Advia Centaur analyzer using a two-site sandwich immunoassay based on the direct chemi-luminometric technology. The capture antibody is a mouse monoclonal anti-ferritin antibody and the indicator antibody is a goat polyclonal anti-ferritin antibody labeled with acridinium ester. This method was also standardized against the WHO reference material. The relationship between these methods is: y (Centaur) $= 1.166 \times$ (Immuno-1) $+ 2.069$. The concentration of plasma ferritin is expressed in ng/ml.

Determination of α-tocopherol and Retinal Levels in Plasma and α-tocopherol in Erythrocytes

Plasma α-tocopherol and retinol concentrations were determined by using a modification of the procedure of Catignani and Bieri.[23] Aliquots of 50 μl of internal standards (1.2 mg/l retinol acetate and 52.5 mg/l α-tocopheryl acetate in ethanol) were mixed with 100 μl of plasma in vortex. For the extraction of lipids, 200 μl of HPLC-grade *n*-hexane was added and mixed for 1 min. The phases were separated by centrifugation at 550*g* for 5 min. The hexane layer was withdrawn and evaporated by flashing with nitrogen. The residue was redissolved in 50 μl of filtered HPLC-grade methanol. An aliquot of 20 μl of the solvent was then injected into an HPLC system (Hitachi, Tokyo, Japan), which consisted of a model L-6200A intelligent pump, a model L-4200 UV-Visible detector, a model D-6000 interface, and an LC organizer. The column was a 3.9 mm × 30 cm of stainless steel packed with a μBondapak C_{18} matrix. A guard column packed with μBondapak C_{18} was attached to the analytical column. The detector was set at 290 nm with a sensitivity of 0.01 absorbance units, full scale. The mobile phase was 98% HPLC-grade methanol, and the flow rate was adjusted to be 1.2 ml/min. Peak areas in the HPLC chromatogram of the sample were converted to μg of α-tocopherol and retinol by using standard curves constructed with the aforementioned internal standards, respectively. The concentrations of retinol and α-tocopherol in plasma are expressed as μM. The washed erythrocytes were resuspended to a 50% NaCl solution, and left standing in the dark for 10 min. The cell suspension was mixed with an equal volume of 10% PCA to precipitate proteins. After centrifugation, the concentration of α-tocopherol in the supernatant was determined by HPLC as described above. The concentration of erythrocyte α-tocopherol is expressed in μM.

Determination of Plasma Ascorbate

The concentration of plasma ascorbate was measured by the method of Kyaw.[24] To 2.0 ml of

fresh plasma in a centrifuge tube, we slowly added 2.0 ml of a color reagent containing 0.75 M sodium tungstate, 0.75 M disodium hydrogen phosphate and 6.25% sulfuric acid (v/v). After mixing, the reaction mixture was allowed to stand at room temperature for 30 min and then centrifuged at 6000*g* for 15 min. The blue-colored supernatant was transferred to a test tube with a Pasteur pipette without disturbing the precipitate. Absorbance at 700 nm was read against a reagent blank. For each set of analysis, a standard and a reagent blank were run in parallel throughout the procedure. Concentrations of plasma ascorbate are expressed in μM.

Statistical Analysis

Logarithmic transformation of data was used since the original values of the mtDNA copy number and 8-OHdG content in leukocytes showed a non-normal distribution. Linear regression analysis and multi-covariate ANOVA were applied in the comparison of mtDNA copy number with age, cigarette smoking, plasma prooxidants/antioxidants and oxidative damage indices including plasma TBARS and leukocyte 8-OHdG content. Analysis of log-likelihood ratio of logistic regression model and the method of curve estimation were applied in the examination of the relationship between age and mtDNA copy number. Levene test was applied for the analysis of variance homogeneity in the subpopulations of leukocytes including neutrophils, lymphocytes, monocytes, basophils and eosinophils. All the statistical analyses were performed using the SPSS statistical package, version 10.5 (Chicago, IL).

RESULTS

Table I summarizes the demographic and biochemical data of the study subjects. All measured values of the biochemical parameters were within the normal range. No significant difference in the proportion of each leukocyte subpopulation was found by Levene homogeneity test of variance ($P > 0.05$).

Regression Analysis of the Factors Contributory to the Alteration of mtDNA Copy Number and 8-OHdG in Leukocytes

Redox factors in plasma that may affect the mtDNA copy number of leukocytes were analyzed by linear regression. As shown in Table II, age ($P = 0.004$), retinol ($P = 0.005$), non-protein thiols ($P = 0.001$) and ferritin ($P = 0.004$) were the significant factors with positive contributions to the mtDNA copy number in leukocytes. However, we found that α-tocopherol ($P = 0.001$) had a negative effect on the mtDNA copy number in leukocytes. The *P* value and *R* square of full regression model were 0.001 and 0.083, respectively. The results in Table III showed that body mass index (BMI) ($P = 0.001$), ferritin ($P = 0.002$), urate ($P = 0.031$), retinol ($P = 0.001$) and non-protein thiols ($P = 0.001$) were the significant factors with positive contribution to plasma TBARS. However, ascorbate ($P = 0.085$), α-tocopherol ($P = 0.044$) and protein thiols ($P = 0.001$) were found to

TABLE II Linear regression analysis of demographic data and plasma levels of prooxidants/antioxidants in the contribution to the alteration of mtDNA copy number in human leukocytes**

	Beta	*t*	*P*
Demographic data			
Age (year)	0.098	2.902	0.004*↑
Gender (male/female)	−0.017	−0.433	0.665
BMI (kg/m^2)	0.018	0.549	0.583
Smoking	0.036	0.956	0.339
Blood biochemistry			
Leukocyte (10^3/μl)	0.035	1.094	0.274
Glucose (mg/dl)	−0.030	−0.923	0.356
Prooxidants			
Homocysteine (μM)	−0.049	−1.524	0.128
Ferritin (ng/ml)	0.063	1.967	0.049*↑
Antioxidants			
Urate (mg/dl)	0.053	1.533	0.126
Albumin (g/dl)	−0.010	−0.303	0.762
Ascorbate (μM)	−0.022	−0.662	0.508
α-tocopherol (μM)	−0.114	−0.348	0.001*↓
Retinol (μM)	0.091	2.843	0.005*↑
Protein thiols (μM)	−0.055	−1.689	0.092
Non-protein thiols (μM)	0.184	5.759	0.001*↑

BMI, body mass index; *$P < 0.05$; B, standardized coefficients of regressions. **$P = 0.001$, $R^2 = 0.083$. ↑, Positive contribution; ↓, Negative contribution.

TABLE III Linear regression analysis of demographic data and plasma levels of prooxidants/antioxidants in the contribution to TBARS in plasma**

	Beta	*t*	*P*
Demographic data			
Age (year)	−0.025	−0.768	0.443
Gender (male/female)	0.005	0.144	0.886
BMI (kg/m^2)	0.105	3.325	0.001*↑
Smoking	0.037	1.026	0.305
Blood biochemistry			
WBC (10^3/μl)	0.011	0.343	0.732
Glucose (mg/dl)	−0.004	−0.126	0.900
Pro-oxidants			
Homocysteine (μM)	−0.017	−0.544	0.587
Ferritin (ng/ml)	0.095	3.079	0.002*↑
Antioxidants (ng/ml)			
Urate (mg/dl)	0.071	2.165	0.031*↑
Albumin (g/dl)	0.009	0.278	0.781
Ascorbate (μM)	−0.055	−1.722	0.085
α-tocopherol (μM)	−0.063	−2.012	0.044*↓
Retinol (μM)	0.120	3.897	0.001*↑
Protein thiols (μM)	−0.155	−4.906	0.001*↓
Non-protein thiols (μM)	0.240	7.817	0.001*↑

BMI, body mass index; *$P < 0.05$; B, standardized coefficients of regressions. **$P = 0.001$, $R^2 = 0.156$. ↑, Positive contribution; ↓, Negative contribution.

correlate negatively with the concentration of TBARS in plasma. The P value and R square of full regression model were 0.001 and 0.156, respectively.

Effects of Age on mtDNA Copy Number of Leukocytes

Age was found to be one of the significantly positive contributors to the alteration of mtDNA copy number in leukocytes (Table II). The age-related alteration of copy number in human leukocytes seems to be better fitted in the quadratic regression model than the lineal regression model. It was noted that mtDNA copy number was changed with age in a biphasic manner: log (mtDNA copy number) $=$ $0.102 \times \text{age} - 0.001 \times \text{age}^2 - 0.339$ ($P = 0.001$, $r = 0.176$) (Fig. 2). The age range of 48–60 was found to be the region of plateau in the relationship between age and mtDNA copy number upon analysis of the log-likelihood ratio of the logistic regression model.

The Copy Number of mtDNA and Oxidative Stress in Leukocytes and Erythrocytes

There was a proportional increase of mtDNA copy number in relation to the oxidative stress indices including leukocyte 8-OHdG content ($P = 0.003$, $R^2 = 0.057$) and plasma level of TBARS ($P = 0.001$, $R^2 = 0.322$), when the confounding factors of age, sex and BMI were controlled (Figs. 3 and 4). On the other hand, the levels of GSH ($P = 0.046$, $R^2 = 0.041$) and α-tocopherol ($P = 0.033$, $R^2 = 0.049$) in the erythrocyte were positively and negatively correlated with the copy number of mtDNA in leukocytes, respectively (Figs. 5 and 6).

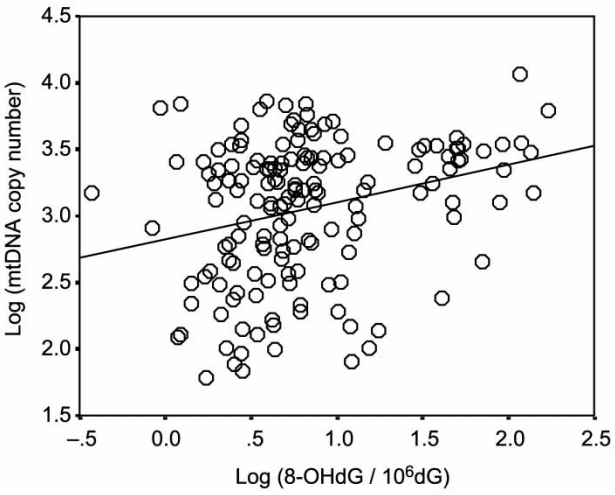

FIGURE 3 Data dispersion and regression analysis of the relationship between 8-hydroxy-2′-deoxyguanosine (8-OHdG) and mtDNA copy number in leukocytes. There are 156 data sets in the scattered plot. Partial correlation analysis was applied under the control of the factors of age, sex, BMI and smoking. $P = 0.003$, $R^2 = 0.057$.

Leukocyte mtDNA Copy Number in Subjects with/without 4,977 bp mtDNA Deletion

In the analysis of multi-covariate ANOVA under the control of the factors of age, sex, BMI and cigarette smoking, we found a significant higher average log (mtDNA copy number) in leukocytes of the subjects harboring 4,977 deletion of mtDNA (3.16 ± 0.15 vs. 3.03 ± 0.11, $P = 0.016$) compared with that of

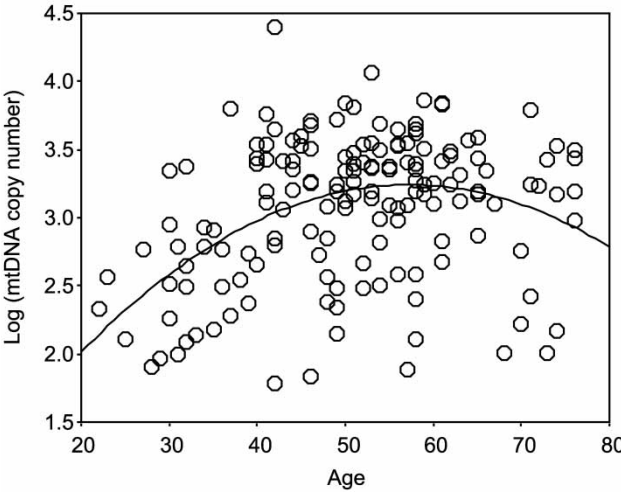

FIGURE 2 Data dispersion and regression analysis of the relationship between age and mtDNA copy number in leukocytes. There are 156 data sets in the scattered plot. The regression curve was fitted in the quadratic regression model: log (mtDNA copy number) $= 0.102 \times \text{age} - 0.001 \times \text{age}^2 - 0.339$, $P = 0.001$, $r = 0.176$.

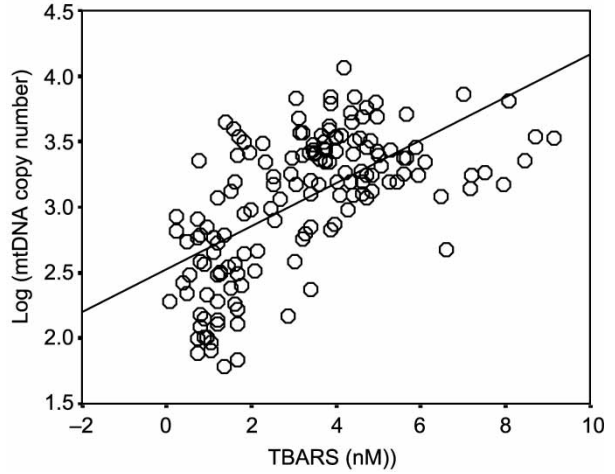

FIGURE 4 Data dispersion and regression analysis of the relationship between TBARS in plasma and mtDNA copy number in leukocytes. There are 156 data sets in the scattered plot. Partial correlation analysis was applied under the control of the factors of age, sex, BMI and smoking. $P = 0.001$, $R^2 = 0.322$.

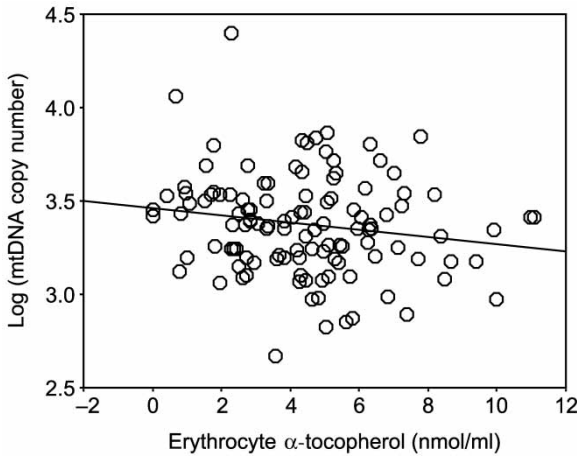

FIGURE 5 Data dispersion and regression analysis of the relationship between erythrocyte α-tocopherol and mtDNA copy number in leukocytes. There are 133 data sets in the scattered plot. Partial correlation analysis was applied under the control of the factors of age, sex, BMI and smoking. $P = 0.033$, $R^2 = 0.049$.

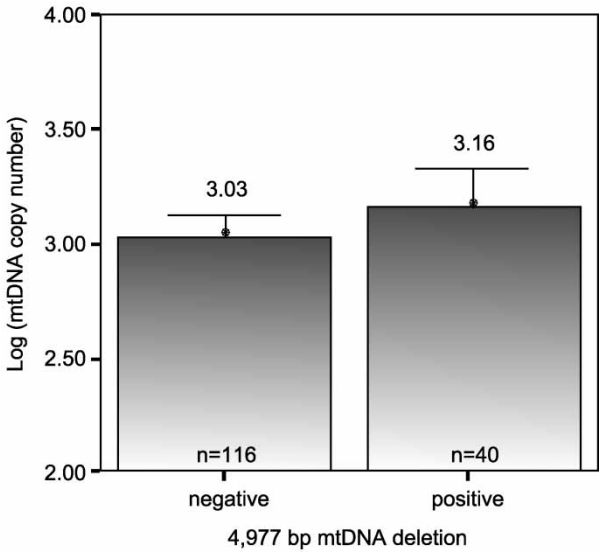

FIGURE 7 Comparison of mtDNA copy number in leukocytes with and without 4,977 bp deletion of mtDNA. Error bar indicates the standard deviation. Multi-covariate ANOVA was performed under the control of age, sex, BMI and smoking, $P = 0.016$.

the subjects who did not harbor such a mtDNA deletion (Fig. 7).

DISCUSSION

Cellular oxidative stress is thought to play a role in the aging process and may affect mtDNA replication in the human cell. In rat splenic lymphocytes, an age-dependent increase of the mitochondrial mass was observed.[25] Increase in the expression of mitochondrial transcription factor A (mtTFA) may be one of such mechanisms involved in this age-related response.[26] In the present study, we found that mtDNA copy number is increased with age by either lineal or quadratic regression.

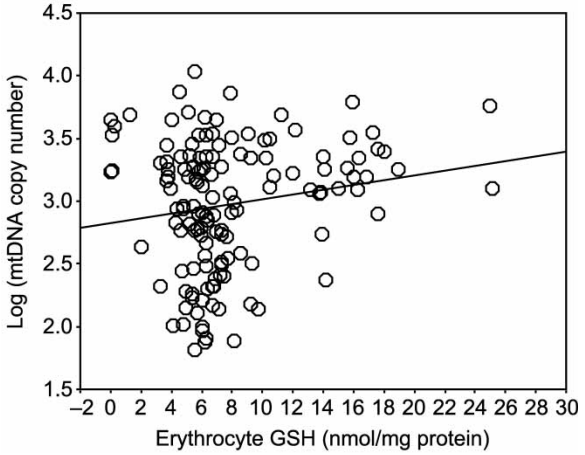

FIGURE 6 Data dispersion and regression analysis of the relationship between erythrocyte GSH and mtDNA copy number in leukocytes. There are 153 data sets in the scattered plot. Partial correlation analysis was applied under the control of the factors of age, sex, BMI and smoking. $P = 0.046$, $R^2 = 0.041$.

However, the relationship between age and mtDNA copy number in leukocytes was better fitted in the quadratic regression model than the lineal model. Before an individual reaches middle age, the mtDNA copy number in the leukocyte seems to correlate positively with age, and progressively shifts to negative correlation thereafter. Age from 48 to 60 seemed to be a plateau response of mtDNA copy number to aging as revealed by the log-likelihood ratio of curve estimation. This biphasic response in alteration of mtDNA copy number was also demonstrated in the study of neurons,[27] muscle cells,[28,29] and leukocytes and abnormal oxidative stress may be one of the possible etiological factors.[30,31] Therefore, the plateau response of mtDNA copy number of leukocytes after middle age may be attributed to the enhanced oxidative stress in advanced aging process, which may provide a rational explanation of the phenomenon of immunosenescence in the human.

Plasma homocysteine and iron are two of the prooxidants that are involved in the oxidative damage and immune response. Homocysteine can exert its toxic effects on human cells through a wide spectrum of possible mechanisms including enhanced oxidative stress elicited by the increase in production of ROS, binding to nitric oxide, production of homocysteinylated/acylated proteins and potent inhibitors of transmethylation reactions.[32] Moreover, homocysteine thiolactonyl derivatives are believed to function as an electron acceptor in oxygen metabolism and as the binding site for ATP synthase in mitochondria.[33] However, no contribution of plasma

homocysteine to the alteration of leukocyte mtDNA copy number was noted in the present study. This unexpected result may imply that the increase in plasma homocysteine in the study subjects was not high enough to induce adequate response such as triggering mtDNA replication. Storage of an excess of iron in the human body can potentially result in oxidative stress. It has been reported that an excess of plasma iron may cause damages to mitochondrial function and mtDNA by decreasing mitochondrial respiratory control and increasing intracellular levels of prooxidants in polymorphonuclear leukocytes.[34] In patients with Friedreich's ataxia, it was found that an accumulation of iron within the mitochondria could induce apoptosis of neuronal cells.[35] In the present study, we found that plasma ferritin, a marker of total iron storage in the human body, was positively related to either the mtDNA copy number in leukocytes or plasma TBARS (Tables II and III). A positive correlation was also noted between mtDNA copy number in leukocytes and plasma TBARS (Fig. 4). Therefore, the oxidative stress-related increase of mtDNA copy number may be resulted, at least partly, from an elevated level of free irons in plasma. However, the contribution of plasma ferritin to the increase in the level of plasma TBARS may be over-estimated by the contamination of irons released from plasma ferritin itself during sample storage as described by Gutteridge *et al.*[36] The biochemical basis for the relationship between ferritin and the TBARS in plasma should be further investigated by determining the ROS and ferritin levels of fresh blood samples.

Several plasma antioxidants including protein thiols, non-protein thiols, urate, ascorbate and α-tocopherol are known to be powerful ROS scavengers which are essential for the maintenance of a fair redox status in plasma.[37] In the present study, only the plasma levels of α-tocopherol and non-protein thiols were found to show negative and positive correlations with the mtDNA copy number of leukocytes, respectively. Moreover, the mtDNA copy number in the leukocyte was also found to correlate positively with the level of GSH and negatively with the α-tocopherol level in erythrocytes. α-Tocopherol is present in both inner and outer membranes of the mitochondria.[38] It has been demonstrated that administration of α-tocopherol resulted in an elevation of α-tocopherol content in the homogenates of nearly all tissues including their mitochondria with a higher capacity to effectively dispose of harmful ROS and free radicals.[39] α-Tocopherol may suppress mtDNA replication *via* a decrease in the generation of organic free radicals and lipid peroxides in blood circulation, which may also affect gene expression in the nucleus and mitochondria.[40] Therefore, the negative correlation between α-tocopherol and mtDNA copy number in blood may be considered as the result of suppression of mtDNA replication by an alleviation of oxidative stress in leukocytes by the antioxidant. However, the GSH level in the erythrocyte was positively correlated with the mtDNA copy number in leukocytes. Similarly, a lower level of 8-OHdG has been found in GSH-depleted mitochondria and a higher level of 8-OHdG was noted in GSH-rich mitochondria.[41] In one of our previous studies, a higher level of plasma GSH was found in young smokers with high oxidative stress in plasma but not in the subjects without cigarette smoking. Therefore, a higher rate of endogenous GSH production was thought to be responsible for the GSH-related increase of mtDNA copy number in cells exposed to mild oxidative stress.[42] It has been shown that a high reserve and effective regeneration of GSH in plasma or intracellular space can metabolize H_2O_2 *via* the GSH pool sequestered in the mitochondria.[43] Therefore, effective endogenous production of GSH but poor regeneration of α-tocopherol in human blood cells without adequate exogenous supplementation may initiate a compensatory response to protect mitochondrial genome from oxidative damage. In this study, the finding of a positive correlation between mtDNA copy number and GSH in erythrocytes as well as non-protein thiols in plasma in healthy subjects may suggest that an increase of mtDNA copy number may serve as an index of the cell response to oxidative stress.

Retinol, although not a real antioxidant in plasma, is known to play a key role in mammalian development, cell maturation and apoptosis.[44,45] It is one of the lipophilic small organic molecules involved in the preservation of mitochondrial number through activation of genes encoding ATPase 6, retinoic acid receptors and mitochondrial transcription factor A.[46] The high activity of mtDNA replication in leukocytes may explain a greater need of retinol for mtDNA replication to proceed or involves some other unknown oxidative stress-related mechanism.[46] These reports and our findings may suggest that the observed retinol-related increase of mtDNA copy number is a result of the regulation of the replication of mtDNA through a redox-sensitive mechanism.

Leukocyte content of 8-OHdG has been successfully used to elucidate the role of oxidative DNA damage as one of the important etiological factors involved in the pathogenesis of autoimmune diseases.[47] Oxidative stress from physical exercise and smoking can result in an elevation of 8-OHdG levels in tissue cells.[48–50] A high level of 8-OHdG in cellular DNA has been found to correlate very well with a high incidence of age-related 4,977 bp deletion

of mtDNA in the brain of healthy individuals.[51] Consistent with the findings of our previous studies, we found that mtDNA copy number was positively correlated with the oxidative stress indices including 8-OHdG content ($P = 0.003$) and 4,977 bp deletion of mtDNA ($P = 0.016$) in leukocytes (Figs. 3 and 5). These results reconfirmed that alteration of mtDNA copy number in blood cells is highly related to oxidative stress, which is dictated by plasma antioxidants/prooxidants and oxidative stress markers in blood circulation.

Prolonged turnover or half-life of circulating leukocytes in the aging process may be another mechanism involved in age-related increase of mtDNA copy number *via* reducing susceptibility of the leukocytes to apoptosis.[52] In the present study, however, we did not find any significant difference in the distribution of leukocyte subpopulations among healthy subjects. Therefore, the observed change of mtDNA copy number can be regarded as a general response to oxidative stress of all subpopulations of leukocytes. Somatic cells in different tissues may have different mitochondrial mass to cope with different energy demand and different types of tissue may have different rates of mitochondrial turnover, which may also affect mtDNA copy number. Indeed, in another study we observed that the mtDNA copy number is higher in the cells with longer lifespan compared with those with shorter lifespan (data not shown).

In summary, the alteration of leukocyte mtDNA copy number is correlated with oxidative stress dictated by the change of levels of antioxidants/prooxidants in the aging process. Ferritin, retinol, non-protein thiols, α-tocopherol in plasma and GSH and α-tocopherol in erythrocytes are six redox factors that were found to correlate significantly with the copy number of mtDNA in leukocytes. These correlations may provide useful information in the future study on the relationships among plasma levels of antioxidants and prooxidants, mtDNA copy number and cellular/humoral immunity. Further studies on oxidative stress-related alteration of mtDNA copy number in different subpopulations of leukocytes and oxidative stress-related alteration of gene expression are also warranted for a better understanding of the redox-related mechanisms involved in the modulation of mtDNA copy number in blood cells.

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