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An *OGG1* polymorphism is associated with mitochondrial DNA content in pesticide-exposed fruit growers

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ABSTRACT

Exposure to pesticides has the capacity to cause mitochondrial dysfunction. An increase mitochondrial DNA (mtDNA) content has also been suggested to relate with DNA damaging agent. In mitochondria, the manganese superoxide dismutase (MnSOD) is a scavenger of reactive oxygen species, and the 8-oxoguanine DNA glycosylase (OGG1) is the major DNA glycosylase for the repair of 8-oxoG lesions. However, the alteration of mtDNA content elicited by pesticide exposure in people with genetic variations in *MnSOD* or *OGG1* has not been investigated. In this study, the mitochondrial to nuclear DNA ratio was quantified in the peripheral blood of 120 fruit growers who experienced pesticide exposure and 106 unexposed controls by real-time quantitative polymerase chain reaction (real-time qPCR). Questionnaires were administered to obtain demographic data and occupational history. The *MnSOD* and *OGG1* genotypes were identified by the PCR based restriction fragment length polymorphism analysis. After adjusting for confounding effects, multiple regression model revealed that subjects experiencing high or low pesticide exposure had a greater mtDNA content than that of controls. The *OGG1* Ser-Ser genotype was also associated with an increased mtDNA content. No association between *MnSOD* genotype and mtDNA content was revealed. Thus, subjects experiencing pesticide exposure had greater mtDNA content and the *OGG1* genotype may modulate mtDNA content in pesticide-exposed fruit growers.

1. Introduction

Pesticides have a very important role in agriculture, and their potential health hazards have been the focus of substantial public interest. Defects in mitochondria have been identified in various cancers (Carew and Huang, 2002) and some degenerative disorders (Orth and Schapira, 2001), and environmental exposure to pesticides has the capacity to cause mitochondrial dysfunction (Yamano and Morita, 1995). Mitochondrial DNA (mtDNA) damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress. This may be due to a high level of oxygen radicals localized at the mitochondrial inner membrane, the lack of efficient mtDNA repair mechanisms, and/or the absence of DNA-binding proteins such as histones (Yakes and Van Houten, 1997). Importantly, changes in mtDNA content have also been suggested to be a response to endogenous and exogenous oxidative stress in human cells (Lee et al., 1998).

Manganese superoxide dismutase (superoxide dismutase 2, SOD2/MnSOD) is a reactive oxygen species (ROS) scavenger in mitochondria (Robinson, 1998). It is synthesized in the cytosol and posttranscriptionally modified for transport into the mitochondrion (Shimoda-Matsubayashi et al., 1996). Within the mitochondria, MnSOD catalyzes the dismutation of superoxide radicals, thereby producing hydrogen peroxide and oxygen. A decrease in MnSOD levels may lead to an imbalance among production and consumption of ROS and free radicals as well as possibly affect to mtDNA. One single nucleotide polymorphism at position −9 of human *MnSOD* (rs4880) has been identified: GTT (valine) to GCT (alanine) (Rosenblum et al., 1996). This polymorphism can alter the

Abbreviations: 8-oxoG, 8-oxoguanine; BER, base excision repair; Ct, cycle number; GLM, general linear model; MnSOD, manganese superoxide dismutase; mtDNA, mitochondrial DNA; OGG1, 8-oxoG glycosylase; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; ROS, reactive oxygen species.

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secondary structure of the protein from a β-pleated sheet to an α-helical conformation (Shimoda-Matsubayashi et al., 1996), and this might affect its cellular localization along with its transport into the mitochondria, leavening mitochondria with an incomplete defense against superoxide radicals.

8-Oxoguanine (8-oxoG) is one of the primary base lesions formed upon oxidative attack against mtDNA in mammalian cells (Bohr et al., 2002). Because 8-oxoG can mispair with adenine, leading to a G:C → A:T transversion, this lesion is highly mutagenic. 8-oxoG is formed in mammalian cells either as a byproduct of normal metabolism or due to oxidative stress generated by exogenous sources, such as pesticides and their metabolites. The 8-oxoG glycosylase (OGG1) protein is the major DNA glycosylase involved in the repair of 8-oxoG lesions (de Souza-Pinto et al., 2001; Kang et al., 1995). A C/G polymorphism at nucleotide position 1245 in exon 7 of the *hOGG1* gene (rs1052133) results in an amino-acid substitution from serine to cysteine at codon 326 (Kim et al., 2003). The repair activity of OGG1 was found to be greater for 326Ser than 326Cys by a functional complementation activity assay of an *Escherichia coli* mutant defective in 8-oxoG repair (Kohno et al., 1998).

In the present study, mtDNA content in the peripheral blood of pesticide-exposed subjects and unexposed controls was compared; and the association of polymorphisms in the *MnSOD* and *OGG1* genes in pesticide-exposed fruit growers with mtDNA content was investigated.

2. Materials and methods

2.1. Study population

The present investigation was a cross-sectional study performed in Tungshin, which is a town located in central Taiwan. The agricultural population of Tungshin is approximately 25,000 people, based on recent population statistics (Taichung County Government, 2009). Traditionally, local farmer associations provide the farmer insurance, finance support, marketing services, and educational training for their members, which consist of commercial and hobby farmers. Citrus, pears, peaches, grapes, persimmons, carambola, and plums constitute more than 95% of the total crop area consisting of 6000 ha. In these farms, pesticides are regularly applied all year round. Airblast sprayers are predominantly used for the application of pesticides. Fruit growing is typically a family business in Tungshin; therefore, exposure is not only limited to the fruit grower, but also to all family members, such as the farmers' wives and children, who often participate in orchard work. During harvesting, hired seasonal workers may also be exposed to crop pesticide residues.

A total of 120 subjects with regular pesticide exposure and 106 unexposed controls who agreed to participate in the present study underwent detailed questionnaire and health examinations. Hired seasonal workers were not included in the current study. An attempt has also been made to minimize possible biases from ethnicity and lifestyle by selecting control subjects originating from the same geographic area and having the same ethnicity of that of the pesticide-exposed subjects. Control occupations included housewives, teachers, clerks, non-farm laborers, skilled workers, small-business persons, and professionals. Among these individuals, none had received any therapeutic irradiation or were taking any medications. All participants were provided with a written description of the study. Those who were unable to read the description had it read to them. Voluntary written consent was obtained from all participants. The study design was approved by the institutional review board of the Chung Shan Medical University, Taichung, Taiwan.

2.2. Epidemiologic information

Information pertaining to personal characteristics was collected from the study subjects using interviewer-administered questionnaires. The structured questionnaire contained questions that covered demographic characteristics and lifestyle, including habits of cigarette smoking, alcohol consumption, and detailed occupational and medical histories. The subject's smoking history included the number of cigarettes smoked daily and the duration of the subject's smoking habit. A variable termed "pack-years" was coined as an indicator of cumulative smoking dose and was defined as the number of packs of cigarettes smoked daily multiplied by the number of years of active smoking. Most Taiwanese farmers have been alerted to the risk of alcohol induced liver damage and have a good understanding that drinking alcohol makes pesticide poisoning worse (Eddleston et al., 2009). In general, alcohol drinking during the period of pesticide application is an unallowable behavior. A concern in the current study was that if pesticide exposed subjects with this condition were included, their prevalence of alcohol drinking would be lower than that of the controls. If there was a substantial difference in mtDNA content between pesticide exposed and control groups, a false association of altered mtDNA content and decreased alcohol drinking might be presented. Therefore, subjects who drink alcohol were removed from the data analysis.

2.3. Assessment of pesticide exposure

Exposure to pesticides consisted of diluting, mixing, loading, and spraying of pesticides as well as maintaining and cleaning the equipment; these tasks were mostly performed by the orchard owner. Other tasks performed in the orchards were bending of branches, thinning of fruit, and pruning. During harvesting, tasks included sorting and transporting fruit, which often required extra labor. For the study, information on past pesticide use by name, amount, area of pesticide application, numbers of treatments per season, years of agrochemical exposure, and use of personal protection equipment was obtained via interviewer-administered questionnaires. Types of work in the orchards were also obtained. The mean orchard size was 1.28 ha (range, 0.06–4.85 ha). The pesticides used by the fruit growers during the preceding 6 months before the medical examination consisted of almost 40 different compounds (Table 1), including organophosphates, carbamates, pyrethroid, insecticides, fungicides, and growth regulators; whereas the application of organochlorines was negligible. On average, each exposed person had applied pesticide about three times a month, with an average cumulative spraying duration of about 7 h/month (range, 2–28 h/month).

Unfortunately, the doses of pesticide exposure could not be calculated for the study subjects due to the lack of environmental monitoring data. Thus, fruit growers were categorized as having low or high pesticide exposure by a modification of the criteria developed by Scarpato et al. (1996): (a) for each subject spraying pesticides, the number of hectares treated was determined and pesticide exposure was calculated by multiplying the average number of treatments × the number of hectares sprayed; (b) the median value of the distribution obtained in (a) was determined, and fruit growers with exposure values less than or greater than the median were assigned to the low or high exposure class, respectively; and (c) subjects who did not directly handle pesticides (e.g., only involved in cutting or harvesting fruits) were considered to have low exposure.

2.4. Copy number of mtDNA

Venous blood was collected into heparinized tubes from all subjects and was separated into plasma, buffy coat, lymphocytes, and red blood cells. These samples were processed the same day and stored at −70 °C. Real-time quantitative polymerase chain reactions (real-time qPCRs) were used to determine the mtDNA copy number in human lymphocytes according to the method developed by Liu et al. (2003) with some modifications. The *ND1* gene (mtDNA) and *β-actin* gene (nuclear DNA) were quantified by real-time qPCR. To evaluate mtDNA copy number, the mitochondrial to nuclear DNA ratio was calculated by dividing the quantity of the *ND1* gene by the corresponding *β-actin* quantity. Primers and the PCR program for the *ND1* gene amplification were also different between our study and the study of Liu et al. The *ND1* gene (264-bp) was amplified using the following primers: forward 5′-GGA GTA ATC CAG GTC GGT-3′ and reverse 5′-TGG GTA CAA TGA GGA GTA GG-3′. The *β-actin* gene (195-bp) was amplified using the following primers: forward 5′-TGG CAT TGC CGA CAG GAT-3′ and reverse 5′-GCT CAG GAG GAG CAA TGA TCT-3′. A total of 20 ng of lymphocyte DNA was used in the real-time qPCR for the determination of the threshold cycle number (Ct) of the nuclear and mitochondrial genes, respectively. Lymphocytes DNA was added to 10 μM of primers and 12.5 μL of 2× SYBR Green PCR Master Mix kit in a final volume of 25 μL. The 2× SYBR Green PCR Master Mix kit supplied by Applied Biosystems (Foster City, CA, USA) contained 250 units AmpliTaq gold DNA polymerase, 100 units AmpEase UNG, 400 μM of dNTP mix with dUTP, 25 mM $MgCl_2$, and 10× SYBR Green PCR buffer. Real-time qPCR was performed using an ABI Prism 7700 Sequence Detector (Applied Biosystems). The PCR program consisted of a 13 min initial denaturation step at 95 °C followed by 40 cycles of 15 s at 95 °C, 15 s at 60 °C, and 45 s at 72 °C. The raw data were processed using the software accompanying the ABI PRISM 7700 Sequencing Detection System (Applied Biosystems). The Ct values for *β-actin* and *ND1* were concurrently determined in each real-time qPCR run and analyzed using ABI Prism SDS Software v9.1 (Applied Biosystems). The efficiency of qPCR was analyzed and standard regression analyses were performed during each run with different amounts of two standard DNA fragments. One was a 264-bp DNA fragment of *ND1* gene, which was amplified from 1.2×10^{-5} to 1.2×10^{1} ng of lymphocytes DNA. The other was a 195-bp DNA fragment of *β-actin* gene amplified from 1.2×10^{-4} to 1.2×10^{2} ng. The experiments were performed in duplicate for each sample. The Ct values were accepted in each qPCR run when correlation efficient was greater than 0.98 and the efficiency was between 1.95 and 2.00. The mean coefficient of variation was 1.35%.

2.5. Genotyping of polymorphic MnSOD and OGG1 genes

Genotyping was performed on genomic DNA extracted from peripheral blood lymphocytes. The determination of the *MnSOD* Ala-9Val (rs4880) genotypes was performed using methods described by Ambrosone et al. (1999). Briefly, for *MnSOD* gene analysis, restriction fragment length polymorphism (RFLP) was detected by differences in *Ngo*MIV site cleavage following PCR amplification. Primers used for the amplification of the *MnSOD* gene were 5′-ACC AGC AGG CAG CTG GCG CCG G-3′ and 5′-GCG TTG ATG TGA GGT TCC AG-3′. A total of 0.5 μL of DNA was added to a

Table 1
The pesticide used by the fruit growers ($n = 120$) during the preceding 6 months before the health examination.

Pesticides	Number of users	Pesticides	Number of users
Organophosphates		Carbamates	
Dimethoate	27 (22.5%)	Methomyl	61 (50.8%)
Omethoate	16 (13.3%)	Carbosulfan	10 (8.3%)
Glyphosate	12 (10.0%)	Carbofuran	8 (6.7%)
Methidathion	11 (9.2%)	Carbaryl	6 (5.0%)
Chlorpyrifos	10 (8.3%)	Benomyl	5 (4.2%)
Methamidophos	4 (3.3%)	Carbendazim	4 (3.3%)
Parathion and parathion-methyl	4 (3.3%)		
Phorate	3 (2.5%)	Fungicides/germicide	
Sumithion	3 (2.5%)	Mancozeb	28 (23.3%)
Phosalone	2 (1.7%)	Tebuconazole	5 (4.2%)
		Thiophanatemethyl	5 (4.2%)
Pyrethroids		Ethylenebisdithiocarbamates	5 (4.2%)
Deltamethrin	14 (11.7%)	Azoxystrobin	3 (2.5%)
Cypermethrin	11 (9.2%)	Oxine-copper	3 (2.5%)
Procymidone	4 (3.3%)	Triadimefon	2 (1.7%)
Permethrin	3 (2.5%)	Thiophanate and thiophanate methyl	1 (0.8%)
Bifenthrin	1 (0.8%)	Propineb	1 (0.8%)
		Chlorothalonil	1 (0.8%)
Insecticides		Iprodion	1 (0.8%)
Imidacloprid	14 (11.7%)	Metalaxyl	1 (0.8%)
Summer oil	8 (6.7%)		
Buprofezin	5 (4.2%)	Growth regulators	6 (5.0%)

reaction containing 200 ng of primers, 1.5 mM $MgCl_2$, 0.2 mM of dNTPs, 50 mM KCl, 10 mM Tris–HCl (pH 8.3), and 0.1% of BSA in a final volume of 50 μL. The PCR cycling parameters consist of a 4 min initial denaturation step at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 61 °C, and 1 min at 72 °C. The reaction was terminated after a final extension of 5 min at 72 °C. The PCR product was digested with *Ngo*MIV, and digestion products were visualized on a 3% agarose gel stained with ethidium bromide. Homozygous Val-Val individuals exhibited a product fragment of 107 bp, whereas homozygous Ala-Ala individuals exhibited an 89 bp and an 18 bp fragment. Heterozygous Val-Ala individuals exhibited all three fragments (Fig. 1a).

OGG1 (rs1052133) genotypes were also determined by PCR-RFLP (Aka et al., 2004). Primers used for the amplification of the *OGG1* gene were 5′-CCC AAC CCC AGT GGA TTC TCA TTG C-3′ and 5′-GGT GCC CCA TCT AGC CTT GCG GCC CTT-3′. After an initial denaturation at 94 °C for 4 min, the cycling conditions were as follows: 35 cycles at 94 °C for 30 s, 61 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 5 min. The PCR products were then digested with *Fnu*4HI overnight at 37 °C and separated on a 2.5% agarose gel. Homozygous Ser-Ser individuals exhibited two product fragments of 213 and 21 bp, whereas homozygous Cys-Cys individuals exhibited a 164 bp, a 49 bp, and a 21 bp fragment. Heterozygous Ser-Cys individuals exhibited all four fragments (Fig. 1b).

2.6. Statistical analysis

Comparisons among low and high pesticide exposure subjects and control subjects regarding age at recruitment, gender, duration of pesticide exposure, size of orchard, current smoking status, and pack-years of smoking were made using Student's *t*-test and ANOVA for continuous variables and the χ^2 test for discrete variables. A χ^2 test or Fisher's exact test was used to test the prevalence of the *MnSOD* and *OGG1* genotypes among low and high pesticide exposure groups and controls. Subsequently, the relative content of mtDNA was evaluated using an analysis stratified by pesticide exposure and different factors. ANOVA was used to compare differences in the relative content of mtDNA by pesticide exposure status, and a Student's *t*-test was used to test the association between the relative content of mtDNA and age, gender, smoking status, and genetic traits. The association of these variables with the relative content of mtDNA was further assessed using a general linear model (GLM). In addition, tests for differences in least squares means were calculated to predict adjusted mtDNA content for study subjects stratified by pesticide exposure status and genotypes. All *p* values were calculated using two-tailed statistical tests, and statistical significance was defined at *p* values <0.05. All data were analyzed using SAS 9.1 software (SAS Institute, Cary, NC, USA).

3. Results

A total of 120 subjects with pesticide exposure and 106 unexposed controls were included in the analysis. The basic characteristics of the study subjects are summarized in Table 2. The mean age was 48.9 ± 1.1 (SE) for unexposed controls, and 56.8 ± 1.5 for individuals experiencing low pesticide exposure, and 56.2 ± 1.4 for those experiencing high pesticide exposure. The proportion of current smokers was 17.7% ($n = 40$). The mean size of the orchards differed significantly between the high and low pesticide exposure groups ($p < 0.01$, *t* test). Age ($p = 0.75$), gender ($p = 0.13$, χ^2 test), duration of pesticide exposure ($p = 0.27$), proportion of current smokers ($p = 0.19$), and cigarette pack-years ($p = 0.16$) did not significantly differ between the high and low pesticide groups. In contrast, the control group was significantly younger in age ($p < 0.01$, ANOVA), the proportion of males was less, and they had fewer pack-years of smoking ($p < 0.01$) than the pesticide-exposed groups.

The genotypic prevalence of *MnSOD* and *OGG1* among the study subjects is shown in Table 3. For all subjects, the frequencies of the Val and Ala alleles of *MnSOD* were 86.9% and 13.1%, respectively. The frequencies of the Ser and Cys alleles of *OGG1* were 37.4%, and 62.6%, respectively. The prevalence of the *MnSOD* ($p = 0.15$, Fisher's exact test) and *OGG1* ($p = 0.23$) genotypes among the low and high pesticide exposure and control groups did not differ significantly. The distributions of all genotypes were in Hardy–Weinberg equilibrium.

The overall mean mtDNA content was 103.2 ± 6.3 (*ND1*:*β-actin* ratio). Table 4 summarizes the crude association of mtDNA content with various factors among test individuals. Individuals experiencing high pesticide exposure (114.3 vs. 88.2; $p = 0.05$, *t*-test) and low pesticide exposure (119.2 vs. 88.2; $p = 0.08$) had greater mtDNA content than those classified as controls. Similarly, individuals with higher pack-years (>10) also had greater mtDNA content compared to those with less pack-years (≤10, 135.6 vs. 98.5; $p = 0.04$). However, a significant difference in mtDNA content was not found between the different gender groups (males vs. females; $p = 0.93$) or age groups (≥53 years [mean age of all subject] vs. <53 years; $p = 0.98$). Interestingly, mtDNA content was found to be greater for individuals featuring the *OGG1* Ser-Ser genotype than those with the Ser-Cys or Cys-Cys genotypes ($p = 0.05$, ANOVA). However, no obvious association between the mtDNA content and the *MnSOD* genotypes was found between the low or high exposure group and controls.

A multiple regression model (GLM) for mtDNA content as a function of age, gender, smoking habit, pesticide exposure, and genotype of *MnSOD* and *OGG1* is shown in Table 5. Because few people had the *MnSOD* Ala-Ala genotype, those with at least one *MnSOD* Ala allele were placed in an *MnSOD* Val-Ala/Ala-Ala genotypes group. In addition, since enzyme activity levels are lower in individuals with at least one *OGG1* Cys allele compared to individ-

Table 2
Basic characteristics of pesticide-exposed fruit growers and controls stratified by different intensities of exposure.

Variables	Controls	Pesticide exposure	
		Low	High
Number of subjects	106 (46.9%)	55 (24.3%)	65 (28.8%)
Mean age (years)	48.9 ± 1.1[a]	56.8 ± 1.5	56.2 ± 1.4[b]
Gender: male (%)	38 (35.9%)	28 (50.9%)	42 (64.6%)[b]
Duration of pesticide exposure (years)	0	28.5 ± 2.4	32.1 ± 2.1
Size of orchard (hectares)	0	0.8 ± 0.1	1.7 ± 0.1[c]
Smoking habit			
Never smoker (%)	88 (83.0%)	44 (80.0%)	44 (67.7%)
Former smoker (%)	4 (3.8%)	1 (1.8%)	5 (7.7%)
Current smoker (%)	14 (13.2%)	10 (18.2%)	16 (24.6%)
Pack-years	2.1 ± 0.6	3.6 ± 1.2	6.4 ± 1.4[b]

[a] Data represent the number of individuals or means ± SE for continuous variables.
[b] $p < 0.01$; comparison among different pesticide-exposure status groups conducted with ANOVA.
[c] $p < 0.01$; comparison between low and high pesticide exposure groups with *t*-test.

Table 3
Prevalence of the *MnSOD* and *OGG1* genotypes among pesticide-exposed fruit growers and controls stratified by pesticide exposure.

	Controls	Pesticide exposure		All
		Low	High	
Number of subjects	106 (46.9%)	55 (24.3%)	65 (28.8%)	226 (100%)[a]
MnSOD				
Val-Val	85 (80.2%)	41 (74.5%)	42 (64.6%)	168 (74.4%)
Val-Ala	20 (18.9%)	14 (25.5%)	23 (35.4%)	57 (25.2%)
Ala-Ala	1 (0.9%)	0 (0%)	0 (0%)	1 (0.4%)
OGG1				
Ser-Ser	12 (11.3%)	6 (10.9%)	8 (12.3%)	26 (11.5%)
Ser-Cys	48 (45.3%)	35 (63.6%)	34 (52.3%)	117 (51.8%)
Cys-Cys	46 (43.4%)	14 (25.5%)	23 (35.4%)	83 (36.7%)

[a] Data represent the numbers of subjects (with percentages in parentheses).

uals with the *OGG1* Ser allele (Ambrosone et al., 1999; Kohno et al., 1998), thus those with at least one *OGG1* Cys allele were combined into an *OGG1* Ser-Cys/Cys-Cys genotypes group. Results revealed that mtDNA content was positively associated with cumulative smoking dose ($p = 0.06$). Relative to controls, an obvious difference in mtDNA content was noted for individuals both in the high ($p = 0.07$) and low ($p = 0.03$) exposure groups. Greater differences in mtDNA content were also observed in individuals harboring the *OGG1* Ser-Ser genotype ($p = 0.03$) compared to the *OGG1* Ser-Cys/Cys-Cys genotype. However, age ($p = 0.49$), gender ($p = 0.34$), and the *MnSOD* genotypes ($p = 0.25$) appeared not to influence the mtDNA content of individuals when examining the data using GLM analysis.

Subsequently, a least squares mean analysis was performed to assess the joint effect on mtDNA content of the *OGG1* polymorphisms and pesticide exposure after adjustment for the effect of

Table 4
Average relative content of mtDNA/nuclear DNA stratified by pesticide-exposure status and various factors.

Variables	Controls		Pesticide exposure				All	
	n	Mean ± SE	Low		High		*n*	Mean ± SE
			n	Mean ± SE	*n*	Mean ± SE		
All	106	88.2 ± 8.2	55	119.2 ± 15.9[a,b]	65	114.3 ± 10.6[b]	226	103.2 ± 6.3
Age								
≥53 years	33	82.4 ± 14.5	34	99.1 ± 13.9	38	125.5 ± 14.3	105	103.4 ± 8.3
<53 years	73	90.8 ± 10.0	21	151.8 ± 34.3	27	98.4 ± 15.4	121	103.1 ± 9.3
Gender								
Males	38	70.8 ± 14.0	28	135.6 ± 23.8	42	109.5 ± 11.2	108	102.6 ± 9.3
Females	68	97.9 ± 9.9	27	102.2 ± 20.8	23	123.0 ± 22.1	118	103.8 ± 8.6
Smoking status								
>10 pack-years	7	75.0 ± 30.6	7	195.1 ± 45.9[b]	15	136.1 ± 22.8	29	135.6 ± 18.8[c]
≤10 pack-years	99	89.1 ± 8.5	48	108.1 ± 16.5	50	107.7 ± 11.9	197	98.5 ± 6.6
MnSOD								
Val-Val	85	90.7 ± 9.3	41	119.8 ± 17.6	42	124.6 ± 13.8	168	106.3 ± 7.3
Val-Ala	20	77.4 ± 17.7	14	117.6 ± 36.2	23	95.5 ± 15.5	57	94.5 ± 12.4
Ala-Ala	1	90.2	0	–	0	–	1	90.2
OGG1								
Ser-Ser	12	131.3 ± 46.8	6	190.6 ± 75.3	8	117.2 ± 24.9	26	140.6 ± 28.1[c]
Ser-Cys	48	94.5 ± 9.9	35	104.8 ± 17.9	34	120.5 ± 16.2	117	105.1 ± 8.2
Cys-Cys	46	70.4 ± 9.7	14	124.6 ± 29.5	23	104.1 ± 16.1	83	88.9 ± 8.8

[a] Comparison among different pesticide-exposure status groups conducted with ANOVA; comparison between age, gender, smoking status, and genotype groups conducted with *t*-test, respectively.
[b] $0.05 < p < 0.10$.
[c] $0.01 < p < 0.05$.

Table 5
Multiple regression model for relative content of mtDNA/nuclear DNA.

Variables	Regression coefficient	SE	*p*-Value
Intercept	91.5	31.3	<0.01
Age: per 1-year increment	−0.4	0.6	0.49
Gender: male vs. female	−13.4	14.0	0.34
Smoking status: >10 vs. ≤10 pack-years	39.0	20.3	0.06
Pesticide exposure			
High vs. control	28.5	15.8	0.07
Low vs. control	34.9	16.1	0.03
Genotyping			
MnSOD: Val-Val vs. Val-Ala/Ala-Ala	16.6	14.3	0.25
OGG1: Ser-Ser vs. Ser-Cys/Cys-Cys	41.6	19.4	0.03

smoking status (Fig. 2). As statistical power was considered, the low and high pesticide exposure groups were combined. In controls, individuals harboring the *OGG1* Ser-Ser genotype had the highest mtDNA content in this group (130.1, $n = 12$), followed by those harboring the *OGG1* Ser-Cys genotype (96.5, $n = 48$). Those harboring the *OGG1* Cys-Cys genotype had the lowest mtDNA content (73.0, $n = 46$). This trend in mtDNA content was shown to be statistically significant with $p = 0.02$ (GLM). The same trend was also shown

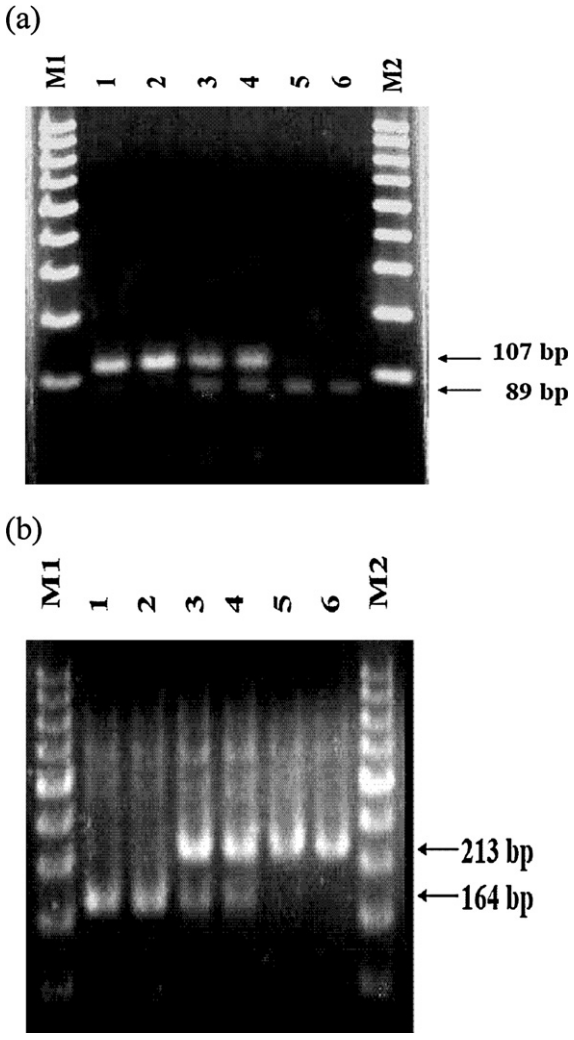

Fig. 1. (a) Representative results of PCR-RFLP analysis of the MnSOD gene. The 107 bp DNA fragment corresponds to the Val allele which does not have a NgoMIV site, while the paired fragments of 107 bp and 89 bp, correspond to the Ala allele containing the NgoMIV site. (b) Representative results of PCR-RFLP analysis of the OGG1 gene. The 213 bp and 21 bp DNA fragment corresponds to the Ser allele, while the fragments of 164 bp, 49 bp and 21 bp, correspond to the Cys allele.

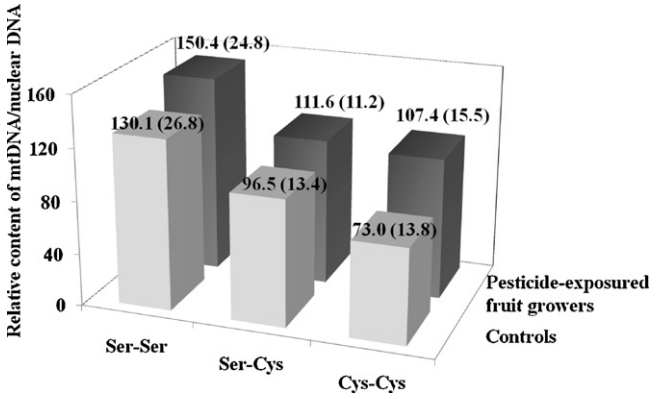

Fig. 2. Relative content of mtDNA/nuclear DNA (β-*actin*) according to the *OGG1* genotypes and pesticide exposure (standard errors in parentheses).

in the pesticide exposure group; however, statistical significance was not observed. Furthermore, among all combined groups of pesticide exposure and *OGG1* genotypes, pesticide exposed subjects with the *OGG1* Ser-Ser genotype had the highest mtDNA content (150.4, $n = 14$), whereas unexposed controls with the *OGG1* Cys-Cys genotype had the lowest mtDNA content (73.0, $n = 37$); this difference was significant ($p < 0.01$).

4. Discussion

Exposure to pesticides has been suggested to be associated with an increased risk of cancer (Zahm et al., 1997) and developing Parkinson's disease (Kamel et al., 2007). Mitochondria defects have also been identified in various cancers (Carew and Huang, 2002) and Parkinson's disease (Orth and Schapira, 2001). Previous studies also demonstrated that exposure to pesticides, inhibitors of mitochondrial complex I, reproduces many features of Parkinson's disease in rats including nigrostriatal dopaminergic degeneration and alpha-synuclein aggregation (Betarbet et al., 2000; Sherer et al., 2003). However, the alteration of mtDNA content elicited by pesticide exposure in people might be modulated by genetic factors. Interestingly, the results in this study revealed an increased mtDNA content in the peripheral blood of individuals exposed to complex mixtures of pesticides and cigarette smoke. The *OGG1* genotype was also associated with an altered content of mtDNA in these subjects.

Previous studies have demonstrated that individuals with susceptible metabolic and repair genotypes experience an increased risk of nuclear DNA damage elicited by pesticide exposure (Liu et al., 2006; Wong et al., 2008). Because human mtDNA has no protective histones and an inefficient DNA repair system, oxidative mtDNA damage is more extensive and persists longer than nuclear DNA damage (Yakes and Van Houten, 1997). In this study, individuals with pesticide exposure also had an increased content in mtDNA. The majority of pesticides used by fruit growers in this study were organophosphates and carbamates. A previous study described the effect of four organophosphorus insecticides on the oxidative processes in rat brain synaptosomes (Sitkiewicz et al., 1980), and the results revealed that higher concentrations of the oxygen analog of ronnel produced inhibition of respiration, cytochrome c oxidase, and NADH–cytochrome c reductase activities. The result in the present study supported the idea that exposure to pesticides may have resulted in oxidative stress, resulting in a decline in mitochondrial function. Furthermore, cells are able to compensate for such injuries by inducing mtDNA replication (Sen and Packer, 1996; Suzuki et al., 1998). In addition, mtDNA content was lower for individuals experiencing high pesticide exposure than those experiencing low pesticide exposure in this study. This suggested that mtDNA under low-oxidative stress may lead to compensatory repli-

cation in response to the increased oxidative stress and functional deterioration of the mitochondria (Sen and Packer, 1996; Suzuki et al., 1998). However, high-oxidative stress may result in damage to the structure of the mtDNA, which may lead to inefficient mtDNA replication (Oka et al., 2008; Shokolenko et al., 2009). Although the response of mitochondria to oxidative stress is well characterized, the mechanism of mtDNA dysfunction caused by pesticide exposure is still unknown. It is also unclear if altered mtDNA content elicited by pesticide exposure could contribute to disease development like Parkinson's disease and cancer. Future studies are necessary to demonstrate the idea presented in the current study.

The current results showed that individuals with the *OGG1* Ser-Ser genotype had the greatest mtDNA content, followed by those harboring the *OGG1* Ser-Cys genotype. Those harboring the *OGG1* Cys-Cys genotype had the lowest mtDNA content. These results suggested that if pesticide-exposed subjects had a greater mtDNA oxidative stress and because individuals harboring the *OGG1* Ser-Cys or Cys-Cys genotype had a lower repair activity than those with the Ser-Ser genotype (Ambrosone et al., 1999; Kohno et al., 1998), compensatory replication of mtDNA might not occur effectively (Banmeyer et al., 2005; Doudican et al., 2005). Although there is no direct evidence for this at present, the degradation of oxidative mtDNA may represent a component of this machinery. Exposure to ROS can result in a number of oxidative modifications to mtDNA, including 8-oxoG. 8-oxoG can be removed from mtDNA through the process of BER by OGG1 protein (de Souza-Pinto et al., 2001). If 8-oxoG is not removed, an A can be inserted opposite of 8-oxoG during replication. The MutY homolog protein can remove the mispaired A opposite 8-oxoG; however, this results in an abasic site which is susceptible to strand breaks. Strand breaks, if not repaired, can lead to DNA degradation (Oka et al., 2008; Shokolenko et al., 2009). In addition, more oxidative damage and a lower energy supply could open the mitochondrial permeability transition pore and lead to apoptosis (Wallace, 1999). These evidences might support the speculation that pesticide-exposed subjects with attenuated DNA repair have an ineffective mtDNA replication. However, the number of subjects in this study who carry Ser-Ser genotype of *OGG1* was relatively small; thus, additional study including more subjects may shed light on this question. Concerning the other key BER enzymes, DNA polymerase γ is the specific polymerase responsible for mtDNA replication (Ogihara et al., 2002). However, polymerase γ may have a dual functionality under low or high levels of oxidative stress. A previous study showed that bright light-induced retinal damage upregulates OGG1 and polymerase γ in photoreceptor synaptic mitochondria (Cortina et al., 2005). In contrast, polymerase γ is also one of the major oxidized mitochondrial matrix proteins, which may result in reduced mtDNA replication and repair capacities (Graziewicz et al., 2002). Therefore, the effect of *OGG1* Ser326Cys polymorphism on mtDNA content in pesticide exposed fruit growers was observed in this study; a possible reason for this is due to individuals suffered to different level of oxidative stress, and affecting the expression of polymerase γ in cellular compensatory mechanism. It is worth speculating on the interaction between polymerase γ and OGG1 on DNA damage elicited by pesticide exposure.

A single nucleotide polymorphism at position −9 of human *MnSOD* has been identified (Rosenblum et al., 1996). This polymorphism might affect its cellular localization along with its transportation into the mitochondria (Shimoda-Matsubayashi et al., 1996). Inefficient targeting of MnSOD could leave mitochondria with an incomplete defense against superoxide radicals. As such, it is possible that oxidative stress may be generated via pesticide metabolism in subjects harboring *MnSOD* variants. However, no significant association between *MnSOD* genotypes and mtDNA content was revealed in the current study. A possible reason for this is that the frequency of the *MnSOD* Ala allele was 13.1%, and the Ala-Ala genotype was not present in the fruit growers enrolled in this study. The possibility of a type II error as a result of insufficient statistical power cannot be ruled out; thus, further study with a larger sample size is required.

In the current study, individuals with higher pack-years of smoking also demonstrated greater mtDNA content. Cigarette smoke contains many carcinogens and a substantial amount of free radical forming substances, such as hydroquinone (Pryor and Stone, 1993). These organic radicals and toxic agents may cause extensive response to mtDNA. Previous studies have also revealed an association between increased mtDNA content and cigarette smoking (Lee et al., 1998; Masayesva et al., 2006). The increase in mtDNA content associated with smoking is thought to be a compensatory mechanism for oxidative stress to mtDNA and respiratory chain components. Furthermore, an association between smoking and increased mtDNA was shown, with persistent alterations in mtDNA content remaining for decades after smoking cessation (Masayesva et al., 2006). Thus, it is possible that the increase in mtDNA was a compensatory consequence of accumulated smoking. Oxidative response to mtDNA has been correlated with the aging process (Lee et al., 1998). However, current study did not reveal a significant association between age and mtDNA content, probably because the age range of subjects was small (mean age, 52.9 ± 0.8).

Although the best source was used to detect pesticide related mtDNA alteration should be its target tissues like liver, kidney, and nerve; such sampling is invasive. The use of peripheral lymphocytes to assess an index of overall mtDNA content should be acceptable because they are long-lived cells (Mohamed et al., 2004). Recently, a significantly positive correlation for mtDNA content between normal colorectal tissues and paired peripheral blood lymphocytes was also observed (Qu et al., 2011). However, further studies would be required to determine the extent of mtDNA copy number in target tissues following pesticide exposure.

In the present study, the frequency of the *MnSOD* Ala allele (13.1%) was consistent with the results of a previous study in Taiwanese adults (13.9%; Cheng et al., 2005). The prevalence of the *OGG1* Ser allele (37.4%) in the current study was quite similar to that previously reported for a Taiwanese population (37.8%; Tarng et al., 2001). The distributions of all genotypes among subjects in this study were in Hardy–Weinberg equilibrium. These findings validated the practice and results of genotyping technique in this study.

Cross-sectional studies such as this have many inherent limitations. The people who participated in this study are generally healthier than those who may have stopped working. It is often difficult to reconstruct an individual's previous pesticide exposure history, including the degree of personal protection used during handing of pesticides. In this study, the available historical exposure data were too sparse and lacking in detail to estimate cumulative exposure. Furthermore, data pertaining to individual exposure were obtained without the knowledge of health outcomes. Consequently, exposure misclassification is assumed to be nondifferential and, if apparent, directed toward an underestimation of the risk for mtDNA alteration. A weakness of the present study is lower age of the control group than that of pesticide exposed fruit growers. This could also affect results of the present study. The current active farm population consisted largely of older people in study area. Most of the younger people have a low regard for agricultural work. Control subjects were not matched to the pesticide-exposed subjects on age. Thus, the control group in the present study was significantly younger in age. This is one of the major lacunae in the current study. Additional study including more appropriate controls would be necessary to provide further evidence. In addition, the role of other antioxidants and DNA repair genes on pesticide-related genotoxicity requires further study.

In summary, subjects experiencing pesticide exposure had greater mtDNA content and the *OGG1* genotype may modulate mtDNA content in pesticide-exposed fruit growers.

Conflict of interest

All authors declare that there is no conflict of interest.

Acknowledgments

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