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Polymorphisms in metabolic *GSTP1* and DNA-repair *XRCC1* genes with an increased risk of DNA damage in pesticide-exposed fruit growers

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ABSTRACT

Pesticide exposure is associated with various neoplastic diseases and congenital malformations. Previous studies have indicated that pesticides may be metabolized by cytochrome P450 3A5 or glutathione *S*-transferases. DNA-repair genes, including X-ray repair cross-complementing group 1 (*XRCC1*) and xeroderma pigmentosum group D (*XPD*), may also be implicated in the process of pesticide-related carcinogenesis. Thus, we investigated whether various metabolic and DNA-repair genotypes increase the risk of DNA damage in pesticide-exposed fruit growers. Using the comet assay, the extent of DNA damage was evaluated in the peripheral blood of 135 pesticide-exposed fruit growers and 106 unexposed controls. The metabolic genotypes *CYP3A5* ($A_{-44}G$) and *GSTP1* (*Ile*105*Val*) and DNA-repair genotypes *XRCC1* (*Arg*399*Gln*, *Arg*194*Trp*, $T_{-77}C$) and *XPD* (*Asp*312*Asn*, *Lys*751*Gln*) were identified by polymerase chain reaction. Our multiple regression model for DNA tail moment showed that age, high pesticide exposure, low pesticide exposure, *GSTP1 Ile-Ile*, and *XRCC1* 399 *Arg-Arg* genotype were associated with increased DNA tail moment (DNA damage). Further analysis of interaction between *GSTP1* and *XRCC1* genes that increase susceptibility revealed a significant difference in DNA tail moment for high pesticide-exposed subjects carrying both *GSTP1 Ile-Ile* with *XRCC1* 399 *Arg-Arg* genotypes (2.49 ± 0.09 μm/cell; $P = 0.004$), compared to those carrying *GSTP1 Ile-Val/Val-Val* with *XRCC1* 399 *Arg-Gln/Gln-Gln* genotypes (1.98 ± 0.15 μm/cell). These results suggest that individuals with susceptible metabolic *GSTP1* and DNA-repair *XRCC1* genotypes may be at increased risk of DNA damage due to pesticide exposure.

1. Introduction

Pesticides have been a boon to developing nations in their efforts to eradicate insect-borne endemic diseases and to produce adequate food and fiber by protecting forests and farms. However, fifty-six pesticides have been classified as carcinogenic to laboratory animals by the International Agency for Research on Cancer [1]. Meta-analyses also showed that pesticide-exposed farmers are at risk for specific tumors including leukemia [2,3] and multiple myeloma [4]. Those individuals at greater risk of developing cancers may possess certain susceptibility factors including inherited metabolic and DNA-repair traits.

Previous studies revealed that organophosphate pesticides, which are most extensively used in Taiwan, are primarily metabolized by hepatic cytochrome P450 3A4 and 3A5 to become an active intermediate organophosphorus-oxon [5,6]. Furthermore, organophosphorus-oxon may then be hydrolyzed by paraoxonase (PON) to diethyl phosphate and 4-nitrophenol [6,7], or conjugated to glutathione (GSH), with subsequent catalysis by glutathione *S*-transferases (GSTs) [8,9]. A previous study performed in Australia also showed that the *GSTP1* gene is associated with an increased risk of Parkinson disease among patients who have been exposed to pesticides [10]. Our previous study has also revealed that *GSTP1* (but not *PON1*, *PON2*, *GSTM1*, and *GSTT1* genotypes) in pesticide-exposed fruit growers is associated with increased DNA damage measured by the comet assay [11]. Although DNA damage (comet assay) was also higher in pesticide-exposed subjects with *CYP3A5* $G_{-44}G$ genotype, the small number of subjects in our previous study has precluded us from drawing a firm conclusion in this regard.

In addition to metabolic traits, DNA-repair capacity also plays an important role in pesticide-related carcinogenesis. Several DNA-repair pathways are known to provide distinct but overlapping

Abbreviations: *GSTP1*, glutathione *S*-transferase P1; *PON1*, paraoxonase 1; *PON2*, paraoxonase 2; *GSTM1*, glutathione *S*-transferase M1; *GSTT1*, glutathione *S*-transferase T1; *CYP3A5*, cytochrome P450 3A5; BER, base excision repair; *XRCC1*, X-ray cross-complementing group 1; NER, nucleotide excision repair; *XPD*, xeroderma pigmentosum group D; GLM, general linear model.

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protection against mutagenic exposures. DNA single-strand breaks are among the most frequent DNA lesions, arising directly from damage to the deoxyribose moieties or indirectly as intermediates of DNA base excision repair (BER) [12–14]. Left unrepaired, DNA single-strand breaks are a major threat to genetic stability and cell survival, accelerating mutation rates and increasing levels of chromosomal aberrations [15–17]. The X-ray cross-complementing group 1 (*XRCC1*) gene product coordinates the actions of DNA polymerase β, DNA ligase III_{α}, and poly (ADP-ribose) polymerase, APE1, polynucleotide kinase/phosphatase, and 8-oxoguanine DNA glycosylase [18–22]. Molecular epidemiological studies have also investigated the possible associations between *XRCC1* polymorphisms and altered cancer risk. The *Arg*399*Gln* (exon 10, base $G \rightarrow A$) is located in the region of the BRCT-I interaction domain of *XRCC1* with poly (ADP-ribose) polymerase, and the *Arg*194*Trp* (exon 6, base $C \rightarrow T$) occurs in the identified proliferating cell nuclear antigen (PCNA) binding region [23,24]. Although measurement of persistence of DNA adducts [25], increased p53 mutations [26], and prolonged cell cycle delay [27] has been used to show the association of 399*Gln* and 194*Trp* variant alleles with some DNA-repair phenotypes, the results from molecular epidemiological studies are still conflicting and rather inconclusive [28]. Recently, a novel *T*-to-*C* transition located at nucleotide −77 in the promoter region of *XRCC1* has been identified, and this substitution was associated with risk of esophageal squamous cell carcinoma in a Chinese population [29]. Bioinformatic analysis suggests that this $T_{-77}C$ polymorphism might disrupt a consensus sequence for Sp1-binding site, implying that this polymorphism could alter *XRCC1* transcription.

The nucleotide excision repair (NER) pathway not only removes and repairs bulky adducts but also may play a role in repair of oxidative DNA damage [30,31]. The xeroderma pigmentosum group D (XPD) protein, a subunit of transcription factor IIH, is an evolutionarily conserved $5' \rightarrow 3'$ helicase that unwinds the DNA in the region of DNA damage. Single nucleotide polymorphisms (SNP) in the *XPD* gene have been studied [28]. *XPD Asp*321*Asn* in exon 10 causes an amino acid substitution in a conserved region of *XPD*. *XPD Lys*751*Gln* in exon 23 also causes an amino acid substitution in the C-terminal part of the protein. The presences of the variant allele *XPD* exon 10 and exon 23 have been associated with relatively high cancer risk in some studies [32,33]. Other studies fail to find statistically significant associations [34,35].

Therefore, pesticide-exposed individuals with inherited, susceptibility-associated, metabolic and DNA-repair genotypes may have increased risk of DNA damage. In this study, we investigated the association of metabolic and DNA-repair genetic polymorphisms in pesticide-exposed fruit growers with cellular DNA damage as measured by the comet assay.

2. Materials and methods

2.1. Study population

Previously, we conducted a study to explore the association between DNA damage and metabolic traits among 91 pesticide-exposed fruit growers and 106 non-exposed controls in Tungshin Town, which is located in central Taiwan. Criteria for selection of the study population were described in detail elsewhere [11]. In current study, sample size was increased to 135 pesticide-exposed fruit growers and 106 non-exposed controls to acquire sufficient statistical discriminatory power to detect a difference in the level of DNA damage. Traditionally, local farmer associations provide farmer insurance, financial support, marketing services, and educational training for their members, who include commercial and hobby farmers. On these farms, pesticides are regularly applied all year. Local farmers who were exposed to pesticides and unexposed controls from the local non-farm population were invited to attend our orientation and participate in our study. We tried to minimize biases due to differences in ethnicity and lifestyle by selecting control subjects who were from the same geographic area and of the same ethnicity as 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. They were also not taking any medications.

2.2. Epidemiologic information

After giving their informed consent, subjects responded to interviewer-administered questionnaires, giving information pertaining to demographic characteristics and lifestyles (including habits of cigarette smoking and alcohol drinking), and detailed occupational and medical histories. The smoking history included the number of cigarettes smoked daily and duration of the smoker's 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 alcohol ingestion aggravates pesticide poisoning. In general, drinking alcohol during the period of pesticide application is not permissible. We therefore were concerned that prevalence of alcohol drinking would be lower in our pesticide-exposed subjects than in our controls. Therefore, all subjects who drank alcohol were excluded.

2.3. Assessment of pesticide exposure

Since exposure to pesticides occurs during diluting, mixing, loading, spraying, maintaining, and cleaning used equipment, 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 questionnaire. The mean orchard size was 1.28 hectares (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, including organophosphates, carbamates, pyrethroid insecticides, fungicides, and growth regulators. Application of organochlorines was negligible. On average, each exposed person reported about three pesticide applications per month with an average cumulative spraying duration of about 7 h/mo (range, 2–28 h/mo).

Unfortunately, levels of pesticide exposure could not be calculated for the study subjects owing to the lack of environmental monitoring data. Thus, we will categorize pesticide exposure as high or low by a modification of the criteria developed by Scarpato et al. [36]: (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 group, respectively; and (c) subjects who did not directly handle pesticides (e.g., those who cut or harvested fruits) were assigned to the low exposure group.

2.4. Comet capture and analysis

Blood samples from pesticide-exposed fruit growers and unexposed controls were collected during a period of extensive pesticide application. Each fruit grower was sampled at the beginning of a mid-week working day. Venous blood was collected in heparinized tubes. The comet assay was conducted under alkali conditions according to Singh et al. [37]. Whole blood (10 μL) was suspended in 1.5% low-melting point agarose and sandwiched between a layer of 0.6% normal-melting agarose and a top layer of 1.5% low-melting point agarose on fully frosted slides. Slides were immersed in lysis solution (1% sodium sarconisate, 2.5 mol/L NaCl, 100 mmol/L Na_2EDTA, 10 mmol/L Tris–HCl, 1% Triton X-100 and DMSO 10%) at 4 °C. After 1 h, slides were placed in electrophoresis buffer (0.3 mmol/L NaOH, 1 mmol/L Na_2EDTA [pH 13]) for 10 min. Electrophoresis was conducted in the same buffer for 15 min at 300 mA. The slides were neutralized with sterilized H_2O thrice for 5 min and stained with 10% ethidium bromide. For each subject, 100 randomly captured comets from slides (25 cells on each of four comet slides) were examined at ×400 magnification using an epifluorescence microscope connected through a black and white camera to an image analysis system (Comet Assay II; Perceptive Instruments Ltd., Haverhill, Suffolk, United Kingdom). The computerized image analysis system acquired images, computed the integrated intensity profiles for each cell, estimated the comet cell components, and evaluated the range of derived parameters. Undamaged cells have an intact nucleus without a tail, and damaged cells have the appearance of a comet. To quantify DNA damage, the tail moment was calculated as the product of the tail length and the fraction of DNA in the comet tail. All slides were scored by one reader who was blind to the status of the subjects.

2.5. Genotyping of polymorphic metabolic and DNA-repair traits

Genomic DNA was extracted from peripheral blood of subjects. Genotyping were analyzed using polymerase chain reaction (PCR)-based methods as described below. The determination of *CYP3A5* $A_{-44}G$ genotypes was done according to Chou et al. [38]. For *CYP3A5* gene analysis, any restriction fragment length polymorphism (RFLP) was detected by differences in FauI sites following PCR amplification. Primers used for

the amplification of the *CYP3A5* gene were 5′-CAG GTG AGA GGA TAT TTA AGA GGC-3′ and 5′-CAT CGC CAC TTG CCT TCT TCA AC-3′. One half microliter of DNA was added to a PCR buffer 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. Amplification was carried out under conditions that the denaturing step was conducted at 94 °C for 30 s, annealing at 65 °C for 30 s, and extension at 72 °C for 45 s. The PCR products were digested with FauI for 24 h at 55 °C. Homozygous $A_{-44}A$ individuals exhibited a product fragment of 337-bp, whereas homozygous $G_{-44}G$ individuals revealed a 232-bp and a 105-bp fragment, and heterozygous $A_{-44}G$ individuals demonstrated all three fragments. *GSTP1-Alw*26I polymorphism was also determined using a PCR-RFLP technique of Harries et al. [39]. An *Ile* to *Val* substitution in exon 5 (codon 105) was amplified to form an undigested fragment of 177 bp using the primer pair 5′-ACC CCA GGG CTC TAT GGG AA-3′ and 5′-TGA GGG CAC AAG AAG CCC CT-3′. Amplification was carried out under conditions that the denaturing step was conducted at 94 °C for 30 s, annealing at 61 °C for 30 s, and extension at 72 °C for 30 s. The PCR products were digested with *Alw*26I for 24 h at 37 °C. Homozygous *Ile-Ile* individuals demonstrated a single product fragment of 177 bp, whereas homozygous *Val-Val* individuals revealed both 92- and 85-bp fragments, and heterozygous *Ile-Val* individuals exhibited all three of the fragments.

The determination of *XRCC1* polymorphism in exon 10 was done as previously described [40]. An *Arg* to *Gln* substitution in exon 10 (codon 399) was amplified to form an undigested fragment of 242 bp using the primer pair 5′-CCC CAA GTA CAG CCA GGT C-3′ and 5′-TGT CCC GCT CCT CTC AGT AG-3′. The amplification was carried out under conditions that denaturing was conducted at 94 °C, annealing at 59 °C, and extension at 72 °C. The PCR products were digested with MspI and analyzed in a 2% agarose gel. Homozygous *Gln-Gln* individuals reflected a single product fragment of 242 bp, whereas homozygous *Arg-Arg* individuals demonstrated both 148- and 94-bp fragments, and heterozygous *Arg-Gln* individuals revealed all three of the fragments. The genotyping assay for the *XRCC1* in exon 6 has been described previously with some modifications [41]. An *Arg* to *Trp* substitution in exon 6 (codon 194) was amplified to form an undigested fragment of 485 bp using the primer pair 5′-GCC AGG GCC CCT CCT TCA A-3′ and 5′-TAC CCT CAG ACC CAC GAG T-3′. The amplification was carried out under conditions that denaturing was conducted at 95 °C for 30 s, annealing at 60 °C for 35 s, and extension at 72 °C for 45 s. The PCR products were digested with *PVU*II and analyzed in a 2% agarose gel. Homozygous *Arg-Arg* individuals reflected a single product fragment of 485 bp, whereas homozygous *Trp-Trp* individuals demonstrated both 396- and 89-bp fragments, and heterozygous *Arg-Trp* individuals revealed all three of the fragments. The *XRCC1* $T_{-77}C$ polymorphism was also detected using the PCR-RFLP assay [29] and the primers 5′-GGG CTG GAG GAA ACG CTC-3′ and 5′-TGG CCA GAA GGA TGA GGT AGA G-3′ to amplify this promoter fragment. The amplification was carried out under conditions that denaturing was conducted at 94 °C for 45 s, annealing at 64 °C for 30 s, and extension at 72 °C for 40 s. The PCR products were digested with BsrBI. Homozygous $C_{-77}C$ individuals reflected a two product fragments of 173-bp and 46-bp, whereas homozygous $T_{-77}T$ individuals demonstrated three 116, 57 and 46-bp fragments, and heterozygous $T_{-77}C$ individuals revealed all four of the fragments.

The *XPD* genotypes were also determined by PCR-RFLP analysis [42]. For amplification of the exon 10 region of *XPD*, which contains the polymorphic StyI restriction site, the oligonucleotide primers used were 5′-CTG TTG GTG GGT GCC CGT ATC TGT TGG TCT-3′ and 5′-TAA TAT CGG GGC TCA CCC TGC AGC ACT TCC T-3′. The amplification was carried out under conditions that denaturing was conducted at 94 °C for 45 s, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min. The PCR products were digested with StyI. Homozygous *Asp-Asp* individuals reflected a two product fragments of 507-bp and 244-bp, whereas homozygous *Asn-Asn* individuals demonstrated three 474, 244 and 33-bp fragments, and heterozygous *Asp-Asn* individuals revealed all four of the fragments. The PCR primers for the *XPD Lys*751*Gln* gene were 5′-GCC CGC TCT GGA TTA TAC G-3′ and 5′-CTA TCA TCT CCT GGC CCC C-3′. The amplification was carried out under conditions that denaturing was conducted at 94 °C for 45 s, annealing at 58 °C for 1 min, and extension at 72 °C for 1 min. The PCR products were digested with PstI. Homozygous *Lys-Lys* individuals reflected a two product fragments of 290-bp and 146-bp, whereas homozygous *Gln-Gln* individuals demonstrated three 227, 146 and 63-bp fragments, and heterozygous *Lys-Gln* individuals revealed all four of the fragments.

All genotyping reading was done blinded to exposure status of study subjects. Rigorous quality control procedures were applied throughout the genotyping process. To avoid PCR contamination, reagents for PCR reaction were carefully aliquoted, and each aliquot was used no more than three times. For each assay, a negative control (no DNA template) was added to monitor PCR contamination. Pilot experiments were always conducted to optimize the restriction digestion conditions. After genotyping each genetic polymorphism, ~20% to 25% of the samples in each genotype group were randomly selected for repeated assays to validate the results.

2.6. Statistical analysis

Low and high pesticide exposure subjects and control subjects were compared with respect to age at recruitment, gender, duration of pesticide exposure, size of orchard, current smoking status, and pack-years of smoking using the Student's *t*-test and ANOVA for continuous variables and the χ^2 test for discrete variables. A χ^2 test or Fisher exact test was used to test the prevalence of genotypes of *CYP3A5*, *GSTP1*, *XRCC1*, and *XPD* among low and high pesticide exposure groups and controls. Subsequently, the crude DNA tail moment was evaluated using an analysis stratified by pesticide exposure and different factors. ANOVA and the Student's *t*-test was used to compare difference in DNA tail moment by different pesticide exposure status, and to test the association between the DNA tail moment and age, gender, smoking status, and metabolic and DNA-repair traits. The association of these variables with the DNA tail moment was further assessed using a general linear model (GLM). Finally, a least-squares mean was performed to predict the adjusted DNA tail moment for individuals with different combinations of susceptible genotypes. All data were analyzed using SAS 9.1 (SAS Institute, Cary, NC, USA) and statistical tests were two-sided.

Table 1
Demographic characteristics of pesticide-exposed fruit growers and controls stratified by different intensity of exposure

Variables	Controls	Pesticide exposure	
		Low	High
No. of subjects	106[a]	62	73
Age (years)	48.9 ± 1.1[a]	57.5 ± 1.2	56.8 ± 1.2[b]
Gender: male (%)	38 (35.8%)	30 (48.4%)	51 (69.9%)[b,c]
Duration of pesticide exposure (years)	0	30.0 ± 2.1	32.3 ± 1.8
Size of orchard (ha)	0	0.8 ± 0.1	1.7 ± 0.1[c]
Smoking habit			
Current smoker (%)	14 (13.2%)	11 (17.7%)	16 (21.9%)
Pack-years	2.1 ± 0.6	5.7 ± 1.8	9.9 ± 2.5[b]

[a] Data represent numbers of individuals or mean ± S.E. for continuous variables.
[b] $P < 0.01$; control group significantly different from the high and low pesticide exposure groups.
[c] $P < 0.01$; compared with the low pesticide exposure group.

3. Results

In total, 135 pesticide-exposed subjects and 106 unexposed controls were included in the analysis. Their demographic characteristics are summarized in Table 1. The mean age was 54 years.

Table 2
Prevalence of genotypes of *CYP3A5*, *GSTP1*, *XRCC1*, and *XPD* among pesticide-exposed fruit growers and controls stratified by pesticide exposure

Genotype		Controls	Pesticide exposure	
Gene	Alleles		Low	High
Number of subjects		106	62	73
CYP3A5	$A_{-44}A$	55 (51.9%)	35 (56.5%)	40 (54.8%)
	$A_{-44}G$	41 (38.7%)	23 (37.1%)	30 (41.1%)
	$G_{-44}G$	10 (9.4%)	4 (6.4%)	3 (4.1%)
GSTP1	*Ile-Ile*	56 (52.8%)	40 (64.5%)	49 (67.1%)
	Ile-Val	43 (40.6%)	19 (30.7%)	22 (30.1%)
	Val-Val	7 (6.6%)	3 (4.8%)	2 (2.8%)
XRCC1 194	*Arg-Arg*	55 (51.9%)	22 (35.5%)	42 (57.5%)
	Arg-Trp	44 (41.5%)	35 (56.5%)	28 (38.4%)
	Trp-Trp	7 (6.6%)	5 (8.0%)	3 (4.1%)
XRCC1 399	*Arg-Arg*	66 (62.3%)	38 (61.3%)	40 (54.8%)
	Arg-Gln	35 (33.0%)	22 (35.5%)	24 (32.9%)
	Gln-Gln	5 (4.7%)	2 (3.2%)	9 (12.3%)
$XRCC1_{-77}$	$T_{-77}T$	79 (74.6%)	50 (80.6%)	60 (82.2%)
	$T_{-77}C$	26 (24.5%)	12 (19.4%)	12 (16.4%)
	$C_{-77}C$	1 (0.9%)	0 (0.0%)	1 (1.4%)
XPD 312	*Asp-Asp*	98 (92.5%)	56 (90.3%)	67 (91.8%)
	Asp-Asn	7 (6.6%)	6 (9.7%)	6 (8.2%)
	Asn-Asn	1 (0.9%)	0 (0.0%)	0 (0.0%)
XPD 751	*Lys-Lys*	96 (90.6%)	55 (88.7%)	62 (84.9%)
	Lys-Gln	9 (8.5%)	7 (11.3%)	11 (15.1%)
	Gln-Gln	1 (0.9%)	0 (0.0%)	0 (0.0%)

Data represent the numbers of subjects (with percentage in parentheses, were shown).

Table 3
Dependence of average tail moment per cell (μm) stratified by pesticide-exposure status on various factors

Variables	Controls		Pesticide exposure			
			Low		High	
	n	Mean ± S.E.	*n*	Mean ± S.E.	*n*	Mean ± S.E.
All	106	1.33 ± 0.03	62	2.03 ± 0.05	73	2.31 ± 0.06[a,**]
Age (years)						
≥54	31	1.38 ± 0.07	38	2.07 ± 0.07	44	2.44 ± 0.07[**]
<54	75	1.30 ± 0.03	24	1.97 ± 0.08	29	2.11 ± 0.11
Gender						
Males	38	1.33 ± 0.04	30	2.02 ± 0.08	51	2.29 ± 0.07
Females	68	1.32 ± 0.04	32	2.04 ± 0.07	22	2.36 ± 0.10
Smoking status						
Current smokers	14	1.31 ± 0.05	11	2.01 ± 0.14	16	1.96 ± 0.08[**]
Past smokers	4	1.31 ± 0.13	4	1.95 ± 0.29	8	2.39 ± 0.12
Never smokers	88	1.33 ± 0.03	47	2.04 ± 0.06	49	2.41 ± 0.07
Cumulative smoking dose						
Heavy smokers (≥15 pack-years)[b]	5	1.38 ± 0.13	8	2.04 ± 0.06	17	2.19 ± 0.08
Light smokers (<15 pack-years)	13	1.28 ± 0.04	7	2.00 ± 0.20	7	1.87 ± 0.10
Nonsmokers	99	1.33 ± 0.03	47	2.04 ± 0.06	49	2.41 ± 0.07[**]
CYP3A5						
$A_{-44}A$	55	1.30 ± 0.02	35	1.98 ± 0.07	40	2.34 ± 0.08
$A_{-44}G$	41	1.33 ± 0.05	23	2.07 ± 0.09	30	2.29 ± 0.08
$G_{-44}G$	10	1.44 ± 0.12	4	2.13 ± 0.11	3	2.11 ± 0.34
GSTP1						
Ile-Ile	56	1.37 ± 0.05	40	2.03 ± 0.07	49	2.39 ± 0.06[*]
Ile-Val	43	1.27 ± 0.02	19	1.98 ± 0.07	22	2.17 ± 0.10
Val-Val	7	1.30 ± 0.05	3	2.35 ± 0.31	2	1.77 ± 0.02
XRCC1 194						
Arg-Arg	55	1.33 ± 0.04	22	2.00 ± 0.08	42	2.24 ± 0.08
Arg-Trp	44	1.32 ± 0.04	35	2.01 ± 0.06	28	2.39 ± 0.08
Trp-Trp	7	1.33 ± 0.08	5	2.29 ± 0.33	3	2.51 ± 0.11
XRCC1 399						
Arg-Arg	66	1.34 ± 0.04	38	2.06 ± 0.07	40	2.42 ± 0.07[*]
Arg-Gln	35	1.31 ± 0.03	22	1.99 ± 0.07	24	2.10 ± 0.09
Gln-Gln	5	1.29 ± 0.10	2	1.91 ± 0.23	9	2.39 ± 0.16
$XRCC1_{-77}$						
$T_{-77}T$	79	1.31 ± 0.03	50	2.03 ± 0.06	60	2.35 ± 0.06
$T_{-77}C$	26	1.37 ± 0.07	12	2.01 ± 0.09	12	2.13 ± 0.12
$C_{-77}C$	1	1.33	0	–	1	1.92
XPD 312						
Asp-Asp	98	1.33 ± 0.03	56	2.07 ± 0.05[**]	67	2.31 ± 0.06
Asp-Asn	7	1.30 ± 0.06	6	1.69 ± 0.05	6	2.34 ± 0.19
Asn-Asn	1	1.14	0	–	0	–
XPD 751						
Lys-Lys	96	1.33 ± 0.03	55	2.06 ± 0.06	62	2.31 ± 0.06
Lys-Gln	9	1.26 ± 0.04	7	1.83 ± 0.12	11	2.30 ± 0.13
Gln-Gln	1	1.14	0	–	0	–

[a] Comparison amongst different pesticide-exposure status groups conducted with ANOVA, and comparison between different age, smoking status, and genotype groups conducted with *t*-test and ANOVA, respectively.
[b] Cut point of cumulative smoking dose was determined according to median of cigarette pack-years among all smokers.
[*] $0.01 < P < 0.05$.
[**] $P < 0.01$.

Gender ($P = 0.01$, χ^2 test) and mean size of orchard ($P < 0.01$, *t*-test) but not mean age ($P = 0.68$), duration of pesticide exposure ($P = 0.40$), proportion of current smokers ($P = 0.55$), and cigarette pack-years ($P = 0.17$), differed significantly between the high and low pesticide groups. In contrast, the control group was significantly younger ($P < 0.01$, ANOVA), included more females ($P < 0.01$), and had fewer pack-years of smoking ($P < 0.01$) than the pesticide-exposed groups.

The genotypic prevalence of *CYP3A5*, *GSTP1*, *XRCC1*, and *XPD* is shown in Table 2. The prevalence of *CYP3A5* ($P = 0.72$, Fisher exact test), *GSTP1* ($P = 0.32$), *XRCC1* 194 ($P = 0.12$), *XRCC1* 399 ($P = 0.28$), $XRCC1_{-77}$ ($P = 0.61$), *XPD* 312 ($P = 0.88$), and *XPD* 751 ($P = 0.52$) 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. In our subsequent analysis, since enzyme activity level is lower in individuals with at least one *CYP3A5* A_{-44} allele than individuals with the *CYP3A5* G_{-44} allele [43], we combined those with at least one *CYP3A5* A_{-44} allele into a *CYP3A5* $A_{-44}G/A_{-44}A$ genotypes group. Additionally, since enzyme activity is lower in individuals with at least one *GSTP1 Val* allele than those with the *GSTP1 Ile* allele [44] and since few had the *GSTP1 Val-Val* genotype, those with the *GSTP1 Ile-Val* and *Val-Val* genotypes were combined. As statistical power was considered and the few had *XRCC1* genotypes, subjects possessing *Trp-Trp* and *Arg-Trp*

Table 4
Multiple regression model for tail moment per cell

Variables	Regression coefficient	S.E.	*P*-value
Intercept	0.62	0.16	<0.01
Age: per 1-year increment	0.008	0.002	<0.01
Gender: male vs. female	0.02	0.06	0.80
Smoking status			
Current smokers vs. never smokers	−0.16	0.07	0.04
Past smokers vs. never smokers	−0.07	0.11	0.53
Pesticide exposure			
High vs. control	0.93	0.06	<0.01
Low vs. control	0.63	0.06	<0.01
Genotyping			
CYP3A5: $G_{-44}G$ vs. $A_{-44}A/A_{-44}G$	0.09	0.09	0.31
GSTP1: *Ile-Ile* vs. *Ile-Val/Val-Val*	0.10	0.05	0.04
XRCC1 194: *Arg-Trp/Trp-Trp* vs. *Arg-Arg*	0.05	0.05	0.30
XRCC1 399: *Arg-Arg* vs. *Arg-Gln/Gln-Gln*	0.10	0.05	0.05
$XRCC1_{-77}$: $T_{-77}T$ vs. $T_{-77}C/C_{-77}C$	0.08	0.06	0.19
XPD 312: *Asp-Asp* vs. *Asp-Asn/Asn-Asn*	0.12	0.13	0.35
XPD 751: *Lys-Lys* vs. *Lys-Gln/Gln-Gln*	0.03	0.11	0.82

genotypes in *XRCC1* 194 were combined. Those with at least one *Gln* allele in *XRCC1* 399 were merged into a *XRCC1* 399 *Arg-Gln/Gln-Gln* genotypes group and those with at least one *XRCC1* C_{-77} allele were combined into a *XRCC1* $T_{-77}C/C_{-77}C$ genotypes group. Similarly, because few people had *XPD* 312*Asn-Asn* and 751*Gln-Gln* and because those with at least one *XPD* 321*Asn* allele and *XPD* 751*Gln* allele have a lower capacity for DNA repair [42], those with at least one *XPD* 321*Asn* allele were placed in a *XPD* 312 *Asp-Asn/Asn-Asn* genotypes group and those with at least one *XPD* 751*Gln* allele in a *XPD* 751 *Lys-Gln/Gln-Gln* genotypes group.

Table 3 summarizes the crude association of tail moment with various factors. Individuals exposed to high levels of pesticide had the highest tail moment (2.31 μm/cell) followed by those exposed to low levels (2.03 μm/cell) and controls (1.33 μm/cell; $P < 0.01$, ANOVA). Similarly, individuals older than 54 years (mean age of all subjects) also showed a higher tail moment, especially in the high exposure group (2.44 vs. 2.11 μm/cell; $P < 0.01$, *t*-test). Current smokers in the high exposure group had a lower tail moment compared with former and never smokers ($P < 0.01$). Heavy (≥15 pack-years) and light smokers (<15 pack-years) in the high exposure group had a lower tail moment compared with nonsmokers ($P < 0.01$). However, tail moment was higher for high exposure individuals with *GSTP1 Ile-Ile* genotype (vs. *Ile-Val/Val-Val*, $P = 0.03$) but not in males or those with higher pack-years of smoking. Interestingly, tail moment was higher in the high exposure group with *XRCC1* 399 *Arg-Arg* genotype than the high exposure group with *XRCC1* 399 *Arg-Gln/Gln-Gln* ($P = 0.03$) and higher in the low exposure group with *XPD* 312 *Asp-Asp* genotype than in the low exposure group with *XPD* 312 *Asp-Asn/Asn-Asn* ($P < 0.01$). However, tail moment was not associated with the *CYP3A5*, *XRCC1* 194, $XRCC1_{-77}$, and *XPD* 751 genotypes.

A multiple linear regression model for the relationship between DNA tail moment and age, gender, smoking status, pesticide exposure, and genotypes of *CYP3A5*, *GSTP1*, *XRCC1* 194, *XRCC1* 399, $XRCC1_{-77}$, *XPD* 312, and *XPD* 751 is shown in Table 4. When multiple testing (Bonferroni correction) was taken into consideration, the DNA tail moment was positively associated with increased age ($P < 0.01$), high exposure ($P < 0.01$), and low exposure ($P < 0.01$). Interestingly, greater differences in tail moment were observed among individuals with *GSTP1 Ile-Ile* genotype ($P = 0.04$), and those with *XRCC1* 399 *Arg-Arg* genotype ($P = 0.05$).

Subsequently, a least-squares mean analysis was performed to assess the joint effect on DNA damage of the *GSTP1* and *XRCC1*

Table 5
Adjusted mean tail moment stratified by pesticide-exposure status according to the susceptible *metabolic GSTP1* and DNA-repair *XRCC1* 399 genotypes[a]

Variables	Controls						Low pesticide exposure						High pesticide exposure					
GSTP1 genotype	*Ile-Val/Val-Val*			*Ile-Ile*			*Ile-Val/Val-Val*			*Ile-Ile*			*Ile-Val/Val-Val*			*Ile-Ile*		
	n	Mean ± S.E.	*P*	*n*	Mean ± S.E.	*P*	*n*	Mean ± S.E.	*P*	*n*	Mean ± S.E.	*P*	*n*	Mean ± S.E.	*P*	*n*	Mean ± S.E.	*P*
XRCC1 399 genotype																		
Arg-Arg	31	1.30 ± 0.05	0.63	35	1.37 ± 0.05	0.15	14	2.06 ± 0.12	0.79	24	2.05 ± 0.09	0.84	15	2.25 ± 0.11	0.15	25	2.49 ± 0.09	0.004
Arg-Gln/Gln-Gln	19	1.26 ± 0.06	Ref.	21	1.34 ± 0.06	0.37	8	2.01 ± 0.16	Ref.	16	1.98 ± 0.11	0.87	9	1.98 ± 0.15	Ref.	24	2.28 ± 0.09	0.09

[a] Adjusted for age, gender, and smoking status.

399 polymorphisms and pesticide exposure after adjustment for the effects of age, gender, and smoking status (Table 5). Compared to controls with *GSTP1 Ile-Val/Val-Val* and *XRCC1* 399 *Arg-Gln/Gln-Gln* genotypes (1.26 ± 0.06 μm/cell), controls with *GSTP1 Ile-Ile* and *XRCC1* 399 *Arg-Arg* genotypes had a greater DNA tail moment (1.37 ± 0.06 μm/cell; the increase was 0.11 μm/cell). Notably, relative to the high exposure group with *GSTP1 Ile-Val/Val-Val* and *XRCC1* 399 *Arg-Gln/Gln-Gln* genotypes (1.98 ± 0.15 μm/cell), the high exposure group with both *GSTP1 Ile-Ile* and *XRCC1* 399 *Arg-Arg* had the highest DNA tail moment (2.49 ± 0.09 μm/cell; vs. reference group, $P = 0.004$), while the high exposure group with both *GSTP1 Ile-Ile* and *XRCC1* 399 *Arg-Gln/Gln-Gln* (2.28 ± 0.09 μm/cell, $P = 0.09$) and the one with both *GSTP1 Ile-Val/Val-Val* and *XRCC1* 399 *Arg-Arg* (2.25 ± 0.11 μm/cell, $P = 0.15$) had a moderately increased level of DNA damage.

4. Discussion

It is important to identify the potential genetic susceptibility factors affecting individual responses to carcinogen exposure. In this study, the metabolic *GSTP1* genotype and DNA-repair *XRCC1* 399 genotype, as well as pesticide exposure, were significantly associated with an increased DNA tail moment.

In our study, the frequency of the *CYP3A5* G_{-44} allele (26.6%) was consistent with the results of a previous study in Taiwanese adults (28.2%; Ref. [38]). The prevalence of the *GSTP1* 105*Val* allele (22.4%) in our study appears to be quite similar to that previously reported for Taiwanese populations (18%; Ref. [45]). The frequency of the *XRCC1* 194*Trp* allele (28.4%) and 399*Gln* allele (23.4%) was also comparable to that previously reported for those of Taiwanese descent (194*Trp* allele 27%, 399Gln allele 26%; Ref. [25]). The prevalence of the *XRCC1* C_{-77} allele (11.2%) in our sample was close to that reported for those of Chinese descent (10%; Ref. [29]). In addition, the frequency of the *XPD* 321*Asn* allele (4.4%) and *XPD* 751*Gln* allele (6.0%) was also similar to that reported for those of Chinese descent (321*Asn* allele 6.5%, 751*Gln* allele 8.7%; Ref. [46]). These findings, to some extent, validate the practice and results of our genotyping technique.

The comet assay is a sensitive method of assessing DNA damage. The comet assay of peripheral blood samples in our study and several previous studies has revealed an increase in DNA damage in individuals exposed to complex mixtures of pesticides [47,48]. Furthermore, genetic variability in the enzymes that metabolize agricultural chemicals or repair DNA damage may also be involved in this process. When detoxification and DNA repair are inefficient, metabolic products accumulate and DNA damage persists, contributing to the carcinogenic process.

Previous studies revealed that pesticide-like organophosphates are primarily metabolized by hepatic cytochrome P450 enzymes to active intermediate organophosphorus-oxon [5,6], which may then be hydrolyzed by PON to diethyl phosphate and 4-nitrophenol [6,7], or be conjugated to GSH via catalysis by GSTs [8,9]. However, our previous study was unable to demonstrate any association of *PON1*, *PON2*, *GSTM1*, and *GSTT1* genotypes with DNA damage as measured by the comet assay. Thus, *PON1*, *PON2*, *GSTM1*, and *GSTT1* were not included in current study. A re-evaluation of the effect of *CYP3A5* genotype on DNA damage in pesticide-exposed subjects failed to show a statistically significant association. The most likely reason for this failure was that the number of subjects who carry the $G_{-44}G$ genotype of *CYP3A5* (which is associated with increased susceptibility) is relatively small. Importantly, DNA damage was higher in pesticide-exposed fruit growers with the *GSTP1 Ile-Ile* genotype than in those without this genotype. A previous report also observed that level of benzo(a)pyrene diolepoxide (BPDE)-DNA adducts was higher in *GSTP1 Ile-Ile* carriers than *GSTP1 Ile-Val* and *Val-Val* carriers [45]. Elevated level of DNA damage in the former may reflect their lower level of metabolic activity.

DNA repair is a very important mechanism in protection against gene mutation and cancer initiation. DNA damage could be induced by environmental carcinogens like pesticides and/or through metabolic processes that increase susceptibility. If not repaired, such damage can be converted into gene mutations and genomic instability. In our study, we further investigated whether genetic polymorphisms in *XRCC1* (a protein that plays a central role in BER and single-strand break repair) and in *XPD* (a helicase involved in NER and basal transcription) could increase the risk of DNA damage in pesticide-exposed subjects.

Previous studies have observed that mutant mouse or Chinese hamster ovary cells (EM9 and EM-C11) with no functional XRCC1 protein are hypersensitive to a broad range of DNA damage induced by alkylating agents, reactive oxygen species, or ionizing radiation [17,49,50]. Interestingly, we found that the *XRCC1* 399 *Arg-Arg* genotype was associated with elevated risk of DNA damage in our pesticide-exposed population. In our study, *XRCC1* 194 genotype and *XRCC1*$_{-77}$ genotype were not significantly associated with increased DNA damage and, with regard to the *XPD* gene, neither *Asp*312*Asn* nor *Lys*751*Gln* polymorphisms influenced DNA damage level. XRCC1 protein is exclusively required for DNA BER, strand-break repair, and maintenance of genetic stability [23,24]. The functional consequences of the *XRCC1* polymorphisms are still not known. *XRCC1* 399*Gln* allele has been associated with increased DNA adducts level [25], increased p53 mutations [26], and prolonged cell cycle delay [27]. However, epidemiologic findings have been inconsistent. Interestingly, Hung et al. [51] previously observed that the *XRCC1* 399 *Gln-Gln* genotype was linked with increased risk of tobacco-related cancers among light smokers, but decreased risk among heavy smokers. It is possible that the resulting increased levels of DNA damage from heavy tobacco smoking might give rise to enhanced apoptosis at the time of cell division and would be manifested as a reduced risk of exposure-induced cancer. Such a model has been proposed to explain reduced risks of sunburn-related nonmelanoma skin cancer in homozygotic carriers of the *XRCC1* 399*Gln* variant [52]. No previous studies have examined the potential relationships between DNA-repair gene polymorphisms and DNA damage caused by pesticide exposure. However, our finding of decreased risk of DNA damage among pesticide-exposed subjects with the *XRCC1* 399*Gln* allele suggests the enhancement of apoptosis as a possible mechanism. Little is known concerning the role of *XRCC1* in apoptosis, but unrepaired BER intermediates are clastogenic and may be able to act as a strong trigger of the apoptotic pathway [53]. An animal study has shown that induction of apoptosis following cold brain injury is tightly linked to reduction in *XRCC1* expression [54]. Therefore, reduced efficiency of the XRCC1 protein (a consequence of the 399*Gln* allele) may result in the impaired ability to repair DNA damage, and such cells may be more likely to undergo apoptosis. Another possible explanation is that the effect of the *XRCC1* variant on DNA-repair capacity may differ with type and strength of the DNA damaging exposures. Further studies would be required to test these hypotheses.

We failed to observe any association between the genetic polymorphisms in the *XPD* gene with DNA damage in our pesticide-exposed subjects. Thus, the NER system, a major pathway for repair of bulky DNA damage, might not be involved in repair of DNA damage produced by pesticide. In addition, these findings should be interpreted with caution since it is well known that more than 20 genes are involved in the NER pathway and different combinations of the wild type of one gene and variant of another are possible.

We tried to minimize possible bias due to lifestyle and ethnicity by selecting control subjects from the same geographic area and of the same ethnicity as our pesticide-exposed subjects. However, the active farm population consisted largely of older people in our study area. Most of the younger residents have a low regard for agricultural work. Thus, our control and test subjects were not matched for age and the former were significantly younger. Ideally, pesticide-exposed subjects and unexposed controls should be matched by demographic variables, especially age, to reduce possible selection bias. However, we concern about the sample size in our study, which limits the statistical power to detect a small increase in risk. Thus, our control and test subjects were not matched for age and the former were significantly younger. As expected, older smoking farmers also had more pack-years of smoking than younger. Although adjustment was also performed for age, gender, and smoking status in our multiple regression model, the effect of selection bias might remain. Previous reports also showed that age is associated with increased DNA damage [55,56]. In the present study, older age was also associated with higher DNA tail moment. The higher DNA tail moment in older subjects indicates either an increased susceptibility to damage with older age or a greater accumulation of pesticide or unidentified carcinogens or mutagens with age. In addition, gender was not associated with a higher DNA damage in our study, and no data in the medical literature indicates substantial gender differences. Previous reports, but not the present study, showed that smoking is associated with DNA damage [55]. This is probably due to the fact that the quantity of cigarettes smoked was smaller in the current study than in other studies [47]. Possibly also due to an adaptive response to genotoxicity in smokers, the effect of additional occupational exposure was more pronounced in non-smokers. Thus, non-smokers probably showed a tendency towards a higher mean DNA tail moment than smokers in our high pesticide-exposed groups.

On the farms of our study area, pesticides are regularly applied all year. In the present investigation, blood samples were collected in a single season (March–May) for the study of genetic damage in pesticide-exposed fruit growers and controls. Each fruit grower was sampled at the beginning of a mid-week working day. Thus, increased DNA damage in our tested subjects might result in an accumulation of unrepaired and new DNA lesion. It is often difficult to reconstruct an individual's previous pesticide exposure history, including the degree of personal protection used during handing pesticides. In this study, the available historical exposure data were too sparse and lacking in detail to estimate cumulative exposure. We categorized pesticide exposure as high or low by a modification of the criteria developed by Scarpato et al. [36]. There was a good correlation between individuals' long-term exposure estimated by our exposure model and acetylcholine esterase level. Thus, our estimation for pesticide exposure in this study should be acceptable. Furthermore, data pertaining to individual exposure were obtained without the knowledge of health outcome. Consequently, exposure misclassification is assumed to be non-differential and, if apparent, lead to an underestimation of the risk of DNA damage. Overall, our study was limited by the small numbers of subjects exposed at a substantial level, especially in the analysis of subgroups. Small numbers of subjects in each subgroup limit the conclusions that can be made regarding associations between pesticides and DNA damage in a single study. This is one of the major lacunae in our study. Additional study including more subjects would be necessary to provide further evidence regarding our findings.

In summary, the results revealed that both metabolic *GSTP1* and DNA-repair *XRCC1* genes could modulate DNA damage in pesticide-exposed fruit growers. Further study to determine the relationship of metabolic and DNA-repair genes with cancers caused by pesticide exposure is warranted.

Conflict of interest statement

None.

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