

GSTP1 Genetic Polymorphism Is Associated with a Higher 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. Animal studies also indicated that pesticides may be metabolized by cytochrome P450 3A5 (CYP3A5) enzymes, paraoxonases (PON1 and PON2), or glutathione S-transferases (GSTM1, GSTT1, and GSTP1). However, little is known about the genotoxicity of pesticides in people with various genetic polymorphisms of human CYP3A5, PON1, PON2, GSTM1, GSTT1, and GSTP1. Thus, this study was designed to investigate whether various metabolic genotypes are more susceptible to DNA damage in pesticide-exposed fruit growers. Using the Comet assay, the extent of DNA damage was evaluated in the peripheral blood of 91 fruit growers who experienced pesticide exposure and 106 unexposed controls. Questionnaires were administered to obtain demographic data, cigarette smoking habits, medical, and occupational histories. The genotypes

for CYP3A5, PON1, PON2, GSTM1, GSTT1, and GSTP1 genes were identified by PCR. The results showed that subjects experiencing high or low pesticide exposure had a significantly greater DNA tail moment (DNA damage) than did controls. The multiple regression model also revealed that age ($P < 0.01$), high pesticide exposure ($P < 0.01$), low pesticide-exposure ($P < 0.01$), and CYP3A5 ($P = 0.04$) and GSTP1 ($P = 0.02$) genotypes were significantly associated with an increased DNA tail moment. Further analysis of environmental and genetic interactions revealed a significant interaction for GSTP1 genotypes to influence DNA tail moment for the high pesticide exposure group. These results suggest that individuals with susceptible metabolic GSTP1 genotypes may experience an increased risk of DNA damage elicited by pesticide exposure. (Cancer Epidemiol Biomarkers Prev 2006;15(4):659–66)

Introduction

Pesticides are chemicals used to control agricultural pests, and their widespread use involves the assessment of their potential hazardous effects. Fifty-six pesticides have been classified as carcinogenic to laboratory animals by the IARC (1). Association with cancer have been also reported in human studies for chemicals, such as phenoxy acid herbicides, 2,4,5-trichlorophenoxyacetic acid, lindane, methoxychlor, toxaphene, and several organophosphates. Meta-analyses showed that pesticide-exposed farmers are at risk for specific tumors, including leukemia (2-4) and multiple myeloma (5). However, epidemiologic data on cancer risk in pesticide-exposed farmers are conflicting. For most cancer sites, farmers were found to have lower cancer rates than other people, probably due to the fact that they are healthy workers (6). In addition, earlier studies also proposed a relationship between the incidence of congenital malformations and parent's exposure to pesticides (7, 8). A recent finding also showed that female pesticide-exposed workers in flower greenhouses may have reduced fertility (9).

It is well known that increased genotoxicity in individuals is related to cancer risk and reproductive toxicity. The majority of pesticides have been tested in a wide variety of mutagenicity assays (10-12) and considered as potential chemical mutagens. However, the effective dose in many single tests is generally

very high. As most occupational and environmental exposures are exposure to mixtures of pesticides, the genotoxic potential evaluated on single compounds could not be extrapolated to humans. Hence, the genotoxicologic biomonitoring in human populations is a useful tool to estimate the genetic risk from an integrated exposure to complex mixtures of pesticide. Several cytogenetic assays have been used to evaluate the potential genotoxicity of pesticide exposures in occupationally exposed populations. However, there are reports on positive genotoxic effects in populations exposed to pesticides (13-15) as well as negative findings (16, 17). During the last few years, the alkaline single-cell gel electrophoresis assay, also known as the Comet assay, has increasingly been used in human biomonitoring studies. This assay is a rapid and sensitive tool to show the damaging effects of different compounds on DNA at the individual cell level. Cells with damaged DNA display increased migration of DNA fragments from the nucleus, generating a comet shape (18, 19).

Metabolic polymorphisms have been implicated in chemical exposure-related health effects. However, the exact role of metabolic traits in pesticide-induced genotoxicity remains unclear. Previous studies revealed that organophosphate pesticides, which are most extensively used in Taiwan, are primarily metabolized by hepatic cytochrome P450 3A4 and 3A5 enzymes to become an active intermediate organophosphorus-oxon (20, 21). Furthermore, organophosphorus-oxon may then be hydrolyzed by paraoxonase to diethyl phosphate and 4-nitrophenol (21, 22), or conjugated to glutathione, with subsequent catalysis by glutathione S-transferases (GST; refs. 23, 24). These subsequent metabolites are easily excreted in the urine. Furthermore, the genetic polymorphisms of human CYP3A5 (25), PON1 (26), PON2 (26), GSTM1 (27), GSTT1 (28), and GSTP1 (29) have been identified. Interestingly, the human PON1 gene is reportedly associated with poor reproductive outcome in Chinese pesticide factory

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workers (30). In addition to the *PON1* gene, the *GSTM1* and *GSTT1* genes also influence the frequency of chromosome aberrations in lymphocytes of pesticide-exposed greenhouse workers (31). A previous study done in Australia also showed that the *GSTP1* gene is associated with an increased risk of Parkinson's disease among patients who have been exposed to pesticides (32). However, little is known about whether *CYP3A5*, *PON1*, *PON2*, *GSTM1*, *GSTT1*, and *GSTP1* metabolic genetic polymorphisms modified by exposure to pesticides result in a greater risk of genotoxicity. Pesticide-exposed individuals with inherited susceptible metabolic genotypes may experience an increased risk of genotoxicity.

The present study was undertaken to examine whether increased DNA damage in the Comet assay was associated with pesticide exposure; the effects of inherited polymorphism of metabolic genes on genotoxicity is also evaluated.

Materials and Methods

Study Population. The present investigation was a cross-sectional study in Tungshin Town, which is located in central Taiwan. The agricultural population of Tungshin is ~45,000 people, based on recent population statistics. Citruses, pears, peaches, grapes, persimmons, carambola, and plums constitute >95% of the total crop area of 6,000 ha. Traditionally, local farmer associations provided farmer insurance, finance support, marketing services, and educational training for their members, which consisted of commercial and hobby farmers. On these farms, pesticides are regularly applied all year. Air-blast 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. Family members, such as the farmers' wives and children, often participate in orchard work. During harvesting, hired seasonal workers also may be exposed to crop pesticide residues.

Initially, three training classes of local farmer association were randomly selected for our study. There were 150 members attending our orientation and who were invited to participate as exposed subjects. During our study period, 150 unexposed controls from the local non-farm population who had not been exposed to pesticide were also invited to participate as nonexposed subjects. We tried to minimize some possible biases from ethnicity and lifestyle by selecting control subjects originating from the same geographic area and ethnicity of pesticide-exposed subjects. Control occupations included housewives, teachers, clerks, non-farm laborers, skilled workers, small-business persons, and professionals. 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. Finally, a total of 91 subjects with pesticide exposure and 106 unexposed controls ages >20 years who agreed to participate in our study and underwent detailed questionnaires and our health examination were included in our analysis. Among these individuals, none had received any therapeutic irradiation. They were also not taking any medications.

Epidemiologic Information. Information pertaining to personal characteristics was collected for study subjects using interviewer-administered questionnaires. The structured questionnaire contained questions that covered demographic characteristics and lifestyles, including habits of cigarette smoking, alcohol drinking, 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 of drinking alcohol makes pesticide poisoning worse. In general, alcohol drinking during the period of pesticide application is an unallowable behavior. We concerned that if pesticide-exposed subjects with this condition were included in our study, their prevalence of alcohol drinking would be lower than controls. Therefore, subjects who drank alcohol were removed from the data analysis.

Assessment of Pesticide Exposure. Exposure to pesticides consists of diluting, mixing, loading, spraying, maintaining, and cleaning used equipment. These tasks are mostly done by the orchard owner. Other tasks done in the orchards are bending of branches, thinning of fruit, and pruning. During harvesting, tasks include sorting and transporting fruit, which often requires 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 questionnaire. The mean orchard size was 1.15 ha (range, 0.06-4.17 ha). The pesticides used by the fruit growers during the preceding 6 months before the medical examination consisted of almost 30 different compounds, 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 thrice a month with an average cumulative spraying duration of about 9 h/mo (range, 2-24 h/mo).

Unfortunately, doses of pesticide exposure could not be calculated for the study subjects due to the lack of environmental monitoring data. Thus, we categorized fruit growers as having low or high pesticide exposure by a modification of the criteria developed by Scarpato et al. (33): (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 \times 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., cutting or harvesting fruits) were considered to have low exposure.

Comet Capture and Analysis. The Comet assay was conducted under alkali conditions according to Singh et al. (18). Venous blood was collected in heparinized tubes. Ten microliters of whole blood were 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 sarcosinate, 2.5 mol/L NaCl, 100 mmol/L Na₂EDTA, 10 mmol/L Tris-HCl, 1% Triton X-100, and 10% DMSO) at 4°C. After 1 hour, slides were placed in electrophoresis buffer [0.3 mol/L NaOH, 1 mmol/L Na₂EDTA (pH 13)] for 10 minutes. Electrophoresis was conducted in the same buffer for 15 minutes at 300 mA. The slides were neutralized with sterilized H₂O thrice for 5 minutes 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 $\times 400$ 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). A computerized image analysis system acquires images, computes the integrated intensity profiles for each cell, estimates the comet cell components, and evaluates the range of derived variables. 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.

Genotyping of Polymorphic Metabolic Traits. The determination of *CYP3A5* A₋₄₄G genotypes was done according to Chou et al. (25). Briefly, for *CYP3A5* gene analysis, any RFLP was detected by differences in *FauI* sites following PCR amplification. Primers used for the amplification of the *CYP3A5* gene were 5'-CAGGTGAGAGGATATTTAAGAGGC-3' and 5'-CATCGCCACTTGCCTTCTTCAAC-3'. The determination of *PON1* Gln¹⁹²Arg (A → G) genotypes was done using a PCR-RFLP technique (34). Primers used for the amplification of the *PON1* gene were 5'-TATTGTGCTGTGGGACCTGAG-3' and 5'-CACGCTAAACCCAAATACATCTC-3'. *PON2-DdeI* polymorphism was also determined using a PCR-RFLP technique (26). Primers used for the amplification of the *PON2* gene were 5'-ACATGCATGTACGGTGGTCTTATA-3' and 5'-AGCAATTCATAGATTAATTGTTA-3'. *GSTM1* and *GSTT1* genotypes were determined by coamplification of two genes (27, 28). Primers used for the *GSTM1* gene were 5'-CTGCCCTACTTGATTGATGGG-3' and 5'-CTGGATTGTAGCAGATCATGC-3'. The primers used for the *GSTT1* gene were 5'-TTCCCTTACTGGTCCTCACATCTC-3' and 5'-TCACCGGATCATGGCCAGCA-3'. Amplification of the human β -globin (110 bp) gene was also done as a positive control for each reaction to confirm the presence of amplifiable DNA in the samples. The primers used for β -globin were 5'-ACACAACCTGTGTTCACTAGC-3' and 5'-CAACTTCATCACGTTACC-3'. *GSTP1-Alw26I* polymorphism was also determined using a PCR-RFLP technique of Harries et al. (29). 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'-ACCCAGGGCTCTATGGGAA-3' and 5'-TGAGGGCACAAGAAGCCCT-3'.

Statistical Analysis. Comparisons among low and high pesticide exposure subjects and with control groups subjects regarding age at recruitment, gender, duration of pesticide exposure, size of orchard, current smoking status, and pack-years of smoking were made 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's exact test was used to test the prevalence of genotypes of *CYP3A5*, *PON1*, *PON2*, *GSTM1*, *GSTT1*, and *GSTP1* among low and high pesticide exposure groups and controls. Because individuals possessing at least one *CYP3A5* A₋₄₄ allele have previously been shown to possess a lower enzyme activity level than the *CYP3A5* G₋₄₄ allele (35), those with at least one *CYP3A5* A₋₄₄ allele were grouped as *CYP3A5* A₋₄₄G/A₋₄₄A genotypes. Subjects with at least one *PON1* Gln allele have a lower enzyme activity than those with the *PON1* Arg allele (22); thus, subjects possessing

PON1 Arg-Gln and Gln-Gln genotypes were grouped together. Similarly, individuals featuring the *PON2* genotype with at least one Ser allele showed a lower enzyme activity level than those with a *PON2* Cys allele (36); hence, those with at least one *PON2* Ser allele were grouped as *PON2* Cys-Ser/Ser-Ser. In addition, because individuals with at least one *GSTP1* Val allele also have a lower enzyme activity than those with the *GSTP1* Ile allele (37), and because the number of people with the *GSTP1* Val-Val genotype was very small, *GSTP1* Ile-Val and Val-Val genotypes were combined. Subsequently, the crude DNA tail moment was evaluated using an analysis stratified by pesticide exposure and different factors. ANOVA was used to compare difference in DNA tail moment by different pesticide exposure status, and a Student's *t* test was used to test the association between the DNA tail moment and age, gender, smoking status, and metabolic traits. The association of these variables with the DNA tail moment was further assessed using a general linear model. In addition, the general linear model was also conducted to test for any interaction between pesticide exposure and genetic polymorphisms in DNA tail moment.

Results

Ninety-one subjects with pesticide exposure and 106 unexposed controls were included in the analysis. The demographic characteristics of the study subjects are summarized in Table 1. The mean \pm SE age of the study subjects in high and low pesticide exposure groups were 55.8 \pm 1.7 and 56.7 \pm 1.6 years, respectively. Age ($P = 0.71$), gender ($P = 0.17$, χ^2 test), duration of pesticide exposure ($P = 0.72$), proportion of current smokers ($P = 0.43$), and cigarette pack-years ($P = 0.30$) did not significantly differ between the high and low pesticide groups. Mean size of orchard differed significantly between the high and low pesticide exposure groups ($P < 0.01$, *t* test). In contrast, the control group was significantly younger in age (48.9 \pm 1.1; $P < 0.01$, ANOVA) and had fewer pack-years of smoking ($P < 0.01$) than the pesticide-exposed groups. The genotypic prevalence of *CYP3A5*, *PON1*, *PON2*, *GSTM1*, *GSTT1*, and *GSTP1* among the study subjects is shown in Table 2. The prevalence of *CYP3A5* ($P = 0.30$, Fisher's exact test), *PON1* ($P = 0.10$, χ^2 test), *PON2* ($P = 0.86$), *GSTM1* ($P = 0.12$), *GSTT1* ($P = 0.74$), and *GSTP1* ($P = 0.73$) genotypes among the low and high pesticide exposure and control groups did not differ significantly.

Table 3 summarizes the crude association of tail moment with various factors among test individuals. Individuals experiencing a high pesticide exposure had the highest tail moment (2.35 μ m/cell) followed by those classified as low pesticide exposure (1.92 μ m/cell) and controls (1.33 μ m/cell; $P < 0.01$, ANOVA). Similarly, individuals older than 52 years

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

Variables	Controls	Pesticide exposure	
		Low	High
No. subjects	106*	43	48 [†]
Age (range), y	48.9 \pm 1.1* (21-83)	56.7 \pm 1.6 (38-79)	55.8 \pm 1.7 [†] (28-78)
Gender: male (%)	38 (35.8%)	18 (41.9%)	27 (56.3%)
Duration of pesticide exposure (y)	0	28.5 \pm 2.7	29.8 \pm 2.4 [‡]
Size of orchard (ha)	0	0.7 \pm 0.1	1.7 \pm 0.1 [‡]
Smoking habit			
Current smoker (%)	15 (14.2%)	7 (16.3%)	11 (22.9%)
Pack-years	2.1 \pm 0.6	5.2 \pm 2.2	9.0 \pm 2.9 [†]

*Data represent numbers of individuals or means \pm SE for continuous variables.

[†] $P < 0.01$; control group significantly different from the high and low pesticide exposure groups.

[‡] $P < 0.01$, compared with the low pesticide exposure group.

Table 2. Prevalence of genotypes of *CYP3A5*, *PON1*, *PON2*, *GSTM1*, *GSTT1*, and *GSTP1* among pesticide-exposed fruit growers and controls stratified by pesticide exposure

Gene	Genotype Alleles	Pesticide exposure		
		Controls	Low	High
No. subjects		106	43	48
<i>CYP3A5</i>	<i>A₋₄₄A</i>	55 (51.9%)*	26 (60.5%)	26 (54.2%)
	<i>A₋₄₄G</i>	41 (38.7%)	16 (37.2%)	21 (43.7%)
	<i>G₋₄₄G</i>	10 (9.4%)	1 (2.3%)	1 (2.1%)
<i>PON1</i>	<i>Gln-Gln</i>	43 (40.6%)	12 (27.9%)	21 (43.7%)
	<i>Arg-Gln</i>	34 (32.1%)	13 (30.2%)	19 (39.6%)
	<i>Arg-Arg</i>	29 (27.3%)	18 (41.9%)	8 (16.7%)
<i>PON2</i>	<i>Cys-Cys</i>	2 (1.9%)	2 (4.6%)	1 (2.1%)
	<i>Cys-Ser</i>	32 (30.2%)	11 (25.6%)	15 (31.2%)
	<i>Ser-Ser</i>	72 (67.9%)	30 (69.8%)	32 (66.7%)
<i>GSTM1</i>	Null	67 (63.2%)	23 (53.5%)	22 (45.8%)
	Non-null	39 (36.8%)	20 (46.5%)	26 (54.2%)
<i>GSTT1</i>	Null	46 (43.4%)	20 (46.5%)	24 (50.0%)
	Non-null	60 (56.6%)	23 (53.5%)	24 (50.0%)
<i>GSTP1</i>	<i>Ile-Ile</i>	56 (52.8%)	27 (62.8%)	30 (62.5%)
	<i>Ile-Val</i>	43 (40.6%)	14 (32.6%)	16 (33.3%)
	<i>Val-Val</i>	7 (6.6%)	2 (4.6%)	2 (4.2%)

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

(median age of all subjects) also showed a higher tail moment, especially in the high pesticide exposure group (2.53 versus 2.11 $\mu\text{m}/\text{cell}$; $P < 0.01$, t test). Current smokers in the high pesticide exposure group had a lower tail moment compared with past and never smokers ($P = 0.03$). However, a higher tail moment was not found in males and those with higher pack-years of smoking. Interestingly, the tail moment was found to be higher for individuals in the high pesticide exposure group featuring *GSTP1 Ile-Ile* genotype (versus *Ile-Val/Val-Val*, $P = 0.03$). A higher tail moment was also observed in the high pesticide exposure group with heterozygous *PON1 Arg-Gln* genotype compared with those with high pesticide exposure group with homozygous *PON1 Arg-Arg* genotype ($P = 0.08$). Subjects in the high pesticide exposure group featuring *CYP3A5 G₋₄₄G* genotype also had a higher tail moment than those with *CYP3A5 A₋₄₄G/A₋₄₄A*. However, no obvious association between tail moment and the *PON2*, the *GSTM1*, and the *GSTT1* genotypes was found, and the relationships between tail moment and genotyping were less prominent in the low exposure and control groups.

A multiple regression model (general linear model) for the DNA tail moment as a function of age, gender, smoking habit, pesticide exposure, and genotypes of *CYP3A5*, *PON1*, *PON2*, *GSTM1*, *GSTT1*, *GSTP1* is shown in Table 4. The DNA tail moment was positively associated with an increasing age ($P < 0.01$). Relative to controls, a mean difference in DNA tail moment of 0.98 $\mu\text{m}/\text{cell}$ was noted for individuals experiencing a high pesticide exposure ($P < 0.01$). Individuals classified into the low pesticide exposure group experienced a mean difference of 0.53 $\mu\text{m}/\text{cell}$ compared with controls ($P < 0.01$). Interestingly, greater differences of tail moment were observed among individuals revealing the *CYP3A5 G₋₄₄G* genotype ($P = 0.04$), the *PON2 Cys-Cys* genotype ($P = 0.06$), or the *GSTP1 Ile-Ile* genotype ($P = 0.02$). When multiple testing (Bonferroni correction) was taken into consideration, our results for the *CYP3A5* genotype ($P = 0.09$) and the *PON2* genotype ($P = 0.32$) became nonsignificant, and the *GSTP1* genotype remained significant ($P = 0.04$). However, gender, smoking status, the *PON1* genotype, the *GSTM1* genotype, and the *GSTT1* genotype did not influence the DNA tail moment for individuals when examining the data using a general linear model analysis.

Based on the results of Table 4, the gene-environmental interaction was further investigated after adjusting for age

variable (Table 5). Again, we observed that individuals in the high and low pesticide exposure groups (regardless of genotype) had an increased risk for DNA damage, relative to controls. Although *GSTP1* genotype was not independently associated with an elevated risk for DNA damage, a significantly increased tail moment in DNA of 0.20 $\mu\text{m}/\text{cell}$ was observed in high pesticide-exposed subjects with *GSTP1 Ile-Ile* genotype ($P_{\text{interaction}} = 0.048$). In contrast, no statistically significant interaction was found between the low pesticide exposure and *GSTP1 Ile-Ile* genotype in DNA tail moment ($P_{\text{interaction}} = 0.73$). When we also examined the interaction of pesticide exposure and *CYP3A5* polymorphism, no statistical significance was observed.

Discussion

Genetic biomonitoring of populations exposed to potential carcinogens is a warning system for genetic diseases or cancer. However, there are reports on positive genotoxic effects in populations exposed to pesticides (13-15) as well as negative

Table 3. Average tail moment per cell stratified by pesticide exposure status and various factors

Variables	Controls		Pesticide exposure			
	<i>n</i>	Mean \pm SE	Low		High	
			<i>n</i>	Mean \pm SE	<i>n</i>	Mean \pm SE
All	106	1.33 \pm 0.03	43	1.92 \pm 0.04	48	2.35 \pm 0.06*
Age (y)						
≥ 52	35	1.39 \pm 0.06	28	1.94 \pm 0.05	27	2.53 \pm 0.08 [†]
< 52	71	1.30 \pm 0.03	15	1.88 \pm 0.06	21	2.11 \pm 0.08
Gender						
Male	38	1.33 \pm 0.04	18	1.89 \pm 0.06	27	2.36 \pm 0.08
Female	68	1.32 \pm 0.04	25	1.94 \pm 0.05	21	2.33 \pm 0.10
Smoking status						
Current smokers	15	1.31 \pm 0.05	7	2.01 \pm 0.10	11	2.03 \pm 0.08 [‡]
Past smokers	3	1.31 \pm 0.13	1	1.63	3	2.43 \pm 0.26
Never smokers	88	1.33 \pm 0.03	35	1.91 \pm 0.04	34	2.44 \pm 0.08
Cumulative smoking dose (pack-years)						
> 10	7	1.36 \pm 0.11	6	1.88 \pm 0.10	11	2.20 \pm 0.10
≤ 10	99	1.32 \pm 0.03	37	1.92 \pm 0.04	37	2.39 \pm 0.08
<i>CYP3A5</i>						
<i>G₋₄₄G</i>	10	1.44 \pm 0.12	1	2.34	1	2.79
<i>A₋₄₄G</i>	41	1.33 \pm 0.05	16	1.89 \pm 0.06	21	2.31 \pm 0.10
<i>A₋₄₄A</i>	55	1.30 \pm 0.02	26	1.91 \pm 0.05	26	2.36 \pm 0.09
<i>PON1</i>						
<i>Gln-Gln</i>	43	1.28 \pm 0.02	12	1.93 \pm 0.06	21	2.38 \pm 0.09
<i>Arg-Gln</i>	34	1.35 \pm 0.05	13	1.92 \pm 0.07	19	2.43 \pm 0.10 [§]
<i>Arg-Arg</i>	29	1.37 \pm 0.12	18	1.90 \pm 0.07	8	2.07 \pm 0.18
<i>PON2</i>						
<i>Cys-Cys</i>	2	1.38 \pm 0.17	2	2.02 \pm 0.37	1	2.87
<i>Cys-Ser</i>	32	1.27 \pm 0.03	11	1.99 \pm 0.08	15	2.30 \pm 0.11
<i>Ser-Ser</i>	72	1.35 \pm 0.04	30	1.88 \pm 0.04	32	2.35 \pm 0.08
<i>GSTM1</i>						
Null	67	1.35 \pm 0.04	23	1.91 \pm 0.05	22	2.34 \pm 0.09
Non-null	39	1.29 \pm 0.03	20	1.93 \pm 0.06	26	2.35 \pm 0.09
<i>GSTT1</i>						
Null	46	1.34 \pm 0.05	20	1.96 \pm 0.05	24	2.27 \pm 0.10
Non-null	60	1.32 \pm 0.03	23	1.88 \pm 0.05	24	2.42 \pm 0.08
<i>GSTP1</i>						
<i>Ile-Ile</i>	56	1.37 \pm 0.05	27	1.93 \pm 0.05	30	2.45 \pm 0.07
<i>Ile-Val</i>	43	1.27 \pm 0.02	14	1.87 \pm 0.06	16	2.22 \pm 0.13
<i>Val-Val</i>	7	1.30 \pm 0.05	2	2.09 \pm 0.30	2	1.77 \pm 0.02

NOTE: Values are in $\mu\text{m}/\text{cell}$. Comparison among different pesticide exposure groups is conducted with ANOVA, and comparison between different age, smoking status, and genotype groups is conducted with t test.

* $P < 0.01$; the high exposure group significantly different from the low pesticide exposure and control groups.

[†] $P < 0.01$; compared with individuals ages < 52 years in the high exposure group.

[‡] $P = 0.03$; compared with past and never smokers in the high exposure group.

[§] $P = 0.08$; compared with *PON1 Arg-Arg* genotypes in the high exposure group.

^{||} $P = 0.03$; compared with *GSTP1 Ile-Val/Val-Val* genotypes in the high exposure group.

Table 4. Multiple regression model for tail moment per cell: main effect

Variables	Regression coefficient	SE	P
Intercept	0.82	0.11	<0.01
Age: per 1-y increment	0.008	0.002	<0.01
Gender: male vs female	0.007	0.06	0.90
Smoking status			
Current smokers vs never smokers	-0.08	0.07	0.23
Past smokers vs never smokers	-0.008	0.12	0.94
Pesticide exposure			
High vs control	0.98	0.06	<0.01
Low vs control	0.53	0.06	<0.01
Genotyping			
CYP3A5: G ₋₄₄ G vs A ₋₄₄ G/A ₋₄₄ A	0.19	0.09	0.04
PON1: Arg-Gln/Gln-Gln vs Arg-Arg	0.007	0.05	0.88
PON2: Cys-Cys vs Cys-Ser/Ser-Ser	0.28	0.14	0.06
GSTM1: Null vs non-null	0.03	0.05	0.54
GSTT1: Non-null vs null	0.01	0.04	0.84
GSTP1: Ile-Ile vs Ile-Val/Val-Val	0.11	0.05	0.02

findings (16, 17). The results of the Comet assay presented in this study, together with several previous studies (38, 39), have revealed an increased in DNA damage in the peripheral blood of individuals exposed to complex mixtures of pesticides. The pesticides used by our fruit growers during the preceding 6 months before the medical examination consisted of almost 30 different compounds, including organophosphates. Nevertheless, if the pesticides used by our subjects were not genotoxic, a substantial association between pesticide exposure and DNA tail moment in our Comet assay would be not observed. However, we observed a statistical association between pesticide exposure and DNA tail moment. Our explanation was that our definition of substantial exposure for fruit growers was depended on their number of hectares sprayed and the average number of treatments. The mean duration of pesticide exposure of the exposed subjects was nearly 30 years. This also suggests that our exposed subjects have been exposed to a variety of pesticides. Thus, these definitions of exposure take into account the length of exposure, which may be the important factor in determining risk. The genetic damage shown in the current study (evaluated as an increase in comet tail moment) may originate from DNA single-strand breaks, repair of DNA double-strand breaks, DNA adduct formation, or DNA-DNA and DNA-protein cross-links (40). Environmental exposure to xenobiotics may result in their covalent binding to DNA, which may lead to chromosome alterations, which could be an initial event in the process of chemical carcinogenesis (19). However, the individuals' genetic variability in the enzymes that metabolize agricultural chemicals may also be involved in this process. When these enzymes are not efficient in detoxification, metabolic products accumulate, contributing to the carcinogenic process.

Furthermore, the results of our investigation showed that individuals with CYP3A5 G₋₄₄G or GSTP1 Ile-Ile genotype had a greater DNA tail moment than those with other genotypes. However, it seems that PON1, PON2, GSTM1, and GSTT1 genotypes did not influence the DNA tail moment in the Comet assay among pesticide-exposed fruit growers and control subjects. Importantly, previous studies revealed that pesticide-like organophosphates are primarily metabolized by hepatic cytochrome P450 3A4 and 3A5 enzymes to become active intermediate organophosphorus-oxon (20, 21), which may then be hydrolyzed by PON to diethyl phosphate and 4-nitrophenol (21, 22), or conjugated to glutathione via catalysis by GSTs (23, 24). GSTs metabolize various pesticides, many of which are lipophilic electrophiles (23). Interestingly, in the present study, the GSTP1 Ile-Ile genotype was significantly associated with increased risks for DNA damage, especially in the high pesticide exposure group but not in the low pesticide

exposure group or controls. A recent report also observed increased benzo(a)pyrene diolepoxide/DNA adducts in GSTP1 Ile-Ile carriers when compared with GSTP1 Ile-Val and Val-Val carriers (41). The mechanism for the contrasting effect of GSTP1 genotype remains to be elucidated. The functional effect of the Ile¹⁰⁵ → Val¹⁰⁵ substitution may be substrate dependent. Compared with Ile-containing enzymes, Val-containing GSTP1 is associated with a 7-fold increase in specific activity towards polycyclic aromatic hydrocarbons, but a 3-fold reduction in activity towards 1-chloro-3,4-dinitrobenzene (42, 43). Thus, GSTP1 may have a dual functionality. Adler et al. (44) also suggested that in unstressed conditions, the GSTP1 enzyme acts as a detoxifying enzyme in dimeric form, and that the monomeric form of GSTP1 binds to c-Jun NH₂-terminal kinase, preventing the phosphorylation of c-jun and subsequent apoptosis. Under conditions of stress, the GSTP1 monomer dissociates from c-Jun NH₂-terminal kinase, which subsequently increases the levels of apoptosis. Therefore, under the stress of high-dose pesticide, we hypothesize that GSTP1 Val-containing enzyme is associated with more efficient binding to c-Jun NH₂-terminal kinase, less rapid restoration of kinase activity, and decreased levels of DNA damaged cells elicited by pesticide exposure. Functional studies would be required to test these hypotheses.

In current study, we investigated the genetic role of CYP3A5 but not CYP3A4 in pesticide-related metabolism. CYP3A4 genetic variants have been reported in several populations, but previous studies have failed to find any variant in Chinese (45). CYP3A5 represents at least 50% of the total hepatic cytochrome P450 and metabolizes a wide range of xenobiotics (46). Recently, a A₋₄₄G polymorphism in the promoter of the pseudogene CYP3AP1 has been shown to be linked to the splicing defect of CYP3A5*3, resulting in the absence of CYP3A5 from the tissues in some people (47). Only the subjects with G₋₄₄ in CYP3AP1 had normal CYP3A5 expression. Pesticide-exposed subjects with CYP3A5 G₋₄₄G genotype had a higher DNA damage in the Comet assay, probably because they had a higher CYP3A5 metabolic activity than those with CYP3A5 A₋₄₄G/A₋₄₄A genotypes and therefore an elevated active intermediate levels. By using PCR-RFLP with appropriate endonuclease, the percentage of CYP3A5 G₋₄₄ allele in our study subjects was found to be 25.9%. A previous study has also revealed that the percentage of CYP3A5 G₋₄₄ allele in Chinese (28.2%) is much higher than in Whites (9.2%; ref. 25). This suggests that the CYP3A5 gene may only be a susceptibility gene for pesticide-induced DNA damage in certain ethnic subgroups. Unfortunately, we tested the interaction of pesticide exposure and CYP3A5 polymorphism: no statistical significance was observed. The number of subjects who carry susceptible G₋₄₄G genotype of CYP3A5

Table 5. Multiple regression model for tail moment per cell: gene-environmental interaction

Variables	Regression coefficient	SE	P
Intercept	0.86	0.10	<0.01
Age: per 1-y increment	0.008	0.002	<0.01
Pesticide exposure			
High vs control	0.84	0.08	<0.01
Low vs control	0.55	0.09	<0.01
Genotyping			
<i>CYP3A5</i> : <i>G</i> ₋₄₄ <i>G</i> vs <i>A</i> ₋₄₄ <i>G/A</i> ₋₄₄ <i>A</i>	0.16	0.10	0.18
<i>GSTP1</i> : <i>Ile-Ile</i> vs <i>Ile-Val/Val-Val</i>	0.07	0.05	0.22
Interaction			
High pesticide exposure × <i>CYP3A5 G</i> ₋₄₄ <i>G</i>	0.01	0.32	0.88
Low pesticide exposure × <i>CYP3A5 G</i> ₋₄₄ <i>G</i>	0.24	0.33	0.36
High pesticide exposure × <i>GSTP1 Ile-Ile</i>	0.20	0.10	0.048
Low pesticide exposure × <i>GSTP1 Ile-Ile</i>	-0.04	0.11	0.73

was relatively small; thus, it would seem likely that this was the reason that we observed no significant interaction between pesticide exposure and *CYP3A5* polymorphism in DNA tail moment. Additional study including more subjects may shed light on this question.

Human PON catalyzes the hydrolysis of organophosphates and their metabolites (21, 22). In humans, PON activity varies 10- to 40-fold among individuals (43). Individuals possessing *PON1*¹⁹²*Gln* allele has also been shown to decrease paraoxonase activity (22). Similarly, individuals featuring the *PON2 Ser* allele at codon 311 show a lower enzyme activity level than is the case for those presenting a *PON2 Cys* allele (36). In our study of Taiwanese fruit growers, an elevated DNA damage was more likely to occur in subjects with high pesticide exposure and slow *PON1 Arg-Gln* or *PON2 Ser-Ser* genotype. However, no significant association between *PON1* or *PON2* alone and DNA damage was revealed. Large variation in *PON1* activities within *PON1 Gln*¹⁹²*Arg* genotypes has been observed (48); it is not surprising that the *PON1* activity phenotypes provided additional information about risk of DNA damage in pesticide-exposed subjects that was not provided by genotype alone. In addition, regarding the *PON2 Cys*³¹¹*Ser* polymorphism and DNA damage 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 fact, we also tried to minimize some possible biases from ethnicity and lifestyle by selecting control subjects originating from the same geographic area and ethnicity of pesticide-exposed subjects. However, the current active farm population consisted largely of older people in our study area. Most of the younger people have a low regard for agricultural work. In addition, our control subjects were not matched to the cases on age. Thus, in our study, the control group was significantly younger in age. As expected, older smoking farmers also had more pack-years of smoking than younger. Previous reports also showed that age is associated with DNA damage (49, 50). In the present study of pesticide exposed fruit growers, older age was also associated with a higher DNA tail moment. The higher DNA tail moment in older subjects reflects that there is either an increased susceptibility to damage with age or an accumulation of pesticide or unidentified carcinogens or mutagens. In addition, gender was not associated with a higher DNA damage in our study, and there is no data in the medical literature regarding substantial gender differences. Previous reports show that smoking is associated with DNA damage (50), whereas the present study did not find any positive association between cigarette smoking and DNA damage. This is probably due to the fact that the quantity of cigarettes smoked in the current study was relatively small compared with corresponding figures for participants of other studies (38). Additionally, the genetic polymorphism of

enzymes that metabolize genotoxicants contained in tobacco may influence the results.

The Comet assay is a sensitive method to assess DNA damage (18, 19). However, the major shortcomings of the Comet assay as a tool for biomonitoring studies is the lack of uniformity in Comet assay procedures, such as the duration of alkali unwinding, electrophoresis, and slide scoring. The European Standards Committee on Oxidative DNA Damage (51) has attempted to identify the problems to devise standard, reliable techniques and to reach a consensus on the true background level of damage in normal human cells. However, the fact that variations still occur, even when the standard European Standards Committee on Oxidative DNA Damage protocol is in use. In our study, the mean level (1.33 ± 0.03 μm/cell) of DNA tail moment for our control subjects was similar to that of a previous study for healthy French subjects (1.24 μm/cell; ref. 52). A dispersion coefficient (SD divided by the mean) of 0.24 for our control subjects was likewise consistent with the analogous figures as revealed by previous studies (53). These findings, to some extent, validate the technique of our Comet assay. Rigorous quality control procedures were also applied throughout the genotyping process. To avoid PCR contamination, reagents for PCR reaction were carefully aliquoted, and each aliquot was used no more than thrice. 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. Furthermore, the frequency of the *CYP3A5 G*₋₄₄ allele in our subjects (25.9%) was close to that noted in a previous study pertaining to Taiwanese normal subjects (28.2%; ref. 25). The frequency of *PON1*¹⁹²*Arg* allele in our subjects was 44.7% and was also comparable with the corresponding value for the control group of an atherosclerotic study for the Japanese population (34.8%; ref. 54), whereas it occurred more frequently in sample population from mainland China (62.1%; ref. 30). The frequency of the *PON2*³¹¹*Ser* allele was 82.7%, similarly to that found in a previous study conducted among Chinese controls (82.8%; ref. 55). The prevalence of *GSTM1 null* type (56.9%) and *GSTT1 null* type (45.7%) in our subjects was likewise consistent with previous studies in Taiwan (~50%; ref. 56). Prevalence of *GSTP1*¹⁰⁵*Val* allele (24.1%) in our subjects was also similar to those found in previous studies for Taiwanese (17.6%; ref. 43).

Overall, our study was limited by the relatively 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. Collaborative studies with pooling of rare subtypes are

needed. 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. However, cross-sectional studies such as this have a number of inherent limitations. First, the people who participate in studies are generally healthier than those who may have stopped working. Second, it is often difficult to reconstruct an individual's previous pesticide exposure history, including the degree of personal protection used during handling pesticides. In this study, we deemed the available historical exposure data too sparse and lacking in detail for a quantitative estimation of cumulative exposure. Data pertaining to individual exposure were obtained without the knowledge of health outcome. Consequently, exposure misclassification is assumed to be nondifferential and, if apparent, directed toward an underestimation of the risk for DNA damage.

In summary, the results revealed that metabolic *GSTP1* gene may modulate DNA damage in pesticide-exposed fruit growers. The role of other metabolic genes on pesticide-related genotoxicity requires further study.

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GSTP1 Genetic Polymorphism Is Associated with a Higher Risk of DNA Damage in Pesticide-Exposed Fruit Growers

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