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CYP3A5、PON2 與 GSTT1 基因多形性對於農藥使用者其 DNA 傷害的效應

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CYP3A5 and *GSTP1* Genetic Polymorphisms are Associated with a Higher Risk of DNA Damage in Pesticide-Exposed Fruit Growers[1]

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[3] The abbreviations used are: *CYP3A5*, cytochrome P450 3A5; *PON1*, paraoxonase 1; *PON2*, paraoxonase 2; *GSTM1*, glutathione S-transferase M1; *GSTT1*, glutathione S-transferase T1; *GSTP1*, glutathione S-transferase P1; GLM, general linear model.

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 polymerase chain reaction (PCR). The results showed that subjects experiencing high or low pesticide exposure had a significantly greater DNA tail moment (DAN damage) than did controls. The multiple regression model also revealed that age ($P < 0.01$), high pesticide exposure ($P < 0.01$), low pesticide-exposure ($P < 0.01$), *CYP3A5* ($P = 0.03$), and *GSTP1* ($P = 0.01$) genotypes were significantly associated with an increased DNA tail moment. Further analysis of *CYP3A5* and *GSTP1* gene combinations, revealed an increased trend for these genotypes to influence DNA tail moment for the high pesticide-exposure group. These results suggest that individuals with susceptible metabolic *CYP3A5* and *GSTP1* genotypes may experience an increased risk of DNA damage elicited by pesticide exposure.

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 International Agency for Research on Cancer (IARC) (1). Association with cancer have been also reported in human studies for chemicals such as phenoxy acid herbicides, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), lindane, methoxychlor, toxaphene, and several organophosphates. Meta-analyses showed that pesticide-exposed farmers are at risk for specific tumours including leukaemia (2-4) and multiple myeloma (5). However, epidemiological 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. In addition, earlier studies also proposed a relationship between the incidence of congenital malformations and parent's exposure to pesticides (6, 7). A recent finding also showed that female pesticide-exposed workers in flower greenhouses may have reduced fertility (8).

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 (9-11), 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 genotoxicological 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 (12-14) as well as negative findings (15, 16). During the last few years the

alkaline single cell gel electrophoresis (SCGE) assay, also known as the Comet assay, has increasingly been used in human biomonitoring studies. This assay is a rapid and sensitive tool to demonstrate 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 (17, 18).

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 (19, 20). Furthermore, organophosphorus-oxon may then be hydrolyzed by paraoxonase (PON) to diethyl phosphate and 4-nitrophenol (20, 21), or conjugated to glutathione (GSH), with subsequent catalysis by glutathione S-transferases (GSTs) (22, 23). These subsequent metabolites are easily excreted in the urine. Furthermore, the genetic polymorphisms of human *CYP3A5* [3] (24), *PON1* (25), *PON2* (25), *GSTM1* (26), *GSTT1* (27), and *GSTP1* (28) have been identified. Interestingly, the human *PON1* gene is reportedly associated with poor reproductive outcome in Chinese pesticide factory workers (29). In additional to the *PON1* gene, the *GSTM1* and *GSTT1* genes also influence the frequency of chromosome aberrations in lymphocytes of pesticide-exposed greenhouse workers (30). 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 (31). 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 approximately 45,000 people, based on recent population statistics. Citruses, pears, peaches, grapes, persimmons, carambola, and plums constitute more than 95% of the total crop area of 6,000 hectares. 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 and, 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 from the local farmer association were randomly selected for our study. There were 150 members attending our orientation and, who were invited to participate as potential exposed subjects. During the same study period, 150 unexposed controls from the local non-farm population who had not been exposed to pesticide were also invited to participate as non-exposed subjects. We tried to minimize some possible biases from ethnicity and life-style 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 who aged over 20, 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.

Epidemiological Information. Information pertaining to personal characteristics was collected for study subjects using interviewer-administered questionnaires. The structured questionnaire contained questions that covered demographic characteristics, life styles 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 parameter 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. In general, alcohol drinking during the period of pesticide application is an unallowable behavior, thus 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 performed by the orchard owner. Other tasks performed 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 hectares (range, 0.06–4.17 hectares). 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 three times a month with an average cumulative spraying duration of about 9 h/month (range, 2–24 h/month).

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. (32): 1) 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; 2) the median value of the distribution obtained in (1) 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 3) 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. (17). 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 sarconisate, 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl, 1% Triton X-100 and DMSO 10%) at 4°C. After 1 hour, slides were placed in electrophoresis buffer (0.3 M NaOH, 1 mM Na_2EDTA, pH 13) for 10 minutes. Electrophoresis was conducted in the same buffer for 15 minutes at 300 mA. The slides were neutralized with sterilized H_2O three times for 5 minutes, and then 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 400x magnification using an epifluorescence microscope connected through a black and white camera to an image analysis system (Comet Assay II; Perceptive Instruments Ltd, UK). A computerized image analysis system acquires images, computes the integrated intensity profiles for each cell, estimates the comet cell components, and

then evaluates 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.

Genotyping of Polymorphic Metabolic Traits. The determination of *CYP3A5* $A_{-44}G$ genotypes was performed according to Chou et al. (24). Briefly, for *CYP3A5* gene analysis, any restriction fragment length polymorphism (RFLP) was detected by differences in *Fau*I sites following polymerase chain reaction (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'. The determination of *PON1* Gln192Arg genotypes was performed using a PCR-RFLP technique (33). Primers used for the amplification of the *PON1* gene were 5'-TAT TGT TGC TGT GGG ACC TGA G-3' and 5'-CAC GCT AAA CCC AAA TAC ATC TC-3'. *PON2-Dde*I polymorphism was also determined using a PCR-RFLP technique (25). Primers used for the amplification of the *PON2* gene were 5'- ACA TGC ATG TAC GGT GGT CTT ATA-3' and 5'-AGC AAT TCA TAG ATT AAT TGT TA-3'. *GSTM1* and *GSTT1* genotypes were determined by co-amplification of two genes (26, 27). Primers used for the *GSTM1* gene were 5'-CTG CCC TAC TTG ATT GAT GGG-3' and 5'-CTG GAT TGT AGC AGA TCA TGC-3'. The primers used for the GSTT1 gene were 5'-TTC CTT ACT GGT CCT CAC ATC TC-3' and 5'-TCA CCG GAT CAT GGC CAG CA-3'. Amplification of the human *β-globin* (110-bp) gene was also performed 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'-ACA CAA CTG TGT TCA CTA GC-3' and 5'-CAA CTT CAT CCA CGT TCA CC-3'. *GSTP1-Alw*26I polymorphism was also determined using a PCR-RFLP technique of Harries et al. (28). 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'.

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. Since 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 (34), those with at least one *CYP3A5* A_{-44} allele were grouped as *CYP3A5* $A_{-44}G/A_{-44}A$ genotypes. Subjects with at least one *PON1* Gln allele have a lower enzyme activity than those with the *PON1* Arg allele (21), 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 demonstrated a lower enzyme activity level than those with a *PON2* Cys allele (35), and 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 (36) 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 (GLM). Finally, a least-squares mean was performed to predict the adjusted DNA tail moment for individuals with different numbers of susceptible genotypes. In addition, GLM was also conducted to test for any trend 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 age of the study subjects in high and low pesticide exposure groups were 55.8 ± 1.7 (SE) and 56.7 ± 1.6 years, respectively. Age ($P = 0.71$, *t*-test), 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 ($P < 0.01$). In contrast, the control group was significantly younger in age (48.9 ± 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* amongst 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 amongst test individuals. Individuals experiencing a high pesticide exposure had the highest tail moment (2.35 μm/cell), followed by those classified as low pesticide exposure (1.92 μm/cell), and controls (1.33 μm/cell) ($P < 0.01$, ANOVA). Similarly, individuals older than 52 years of age also showed a higher tail moment, especially in the high pesticide-exposure group (2.53 vs. 2.11 μm/cell; $P < 0.01$, *t*-test). 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 (vs. 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 to those with high pesticide-exposure group with homozygous *PON1* Arg-Arg genotype ($P = 0.08$). Subjects in the high pesticide-exposure group featuring *CYP3A5* $G_{-44}G$ genotype also had a higher tail moment than those with *CYP3A5* $A_{-44}G/A_{-44}A$. 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 (GLM) 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 age greater than 52 years ($P < 0.01$). Relative to controls, a mean difference in DNA tail moment of 1.00 μm/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 0.54 μm/cell compared to controls ($P < 0.01$). Interestingly, greater differences of tail moment were observed amongst individuals revealing the *CYP3A5* $G_{-44}G$ genotype ($P = 0.03$) or the *GSTP1* Ile-Ile genotype ($P = 0.01$). However, gender ($P = 0.85$), smoking status ($P = 0.62$), the *PON1* genotype ($P = 0.79$), the *PON2* genotype ($P = 0.17$), the *GSTM1* genotype ($P = 0.54$), and the *GSTT1* genotype ($P = 0.47$) did not influence the DNA tail moment for individuals when examining the data using a GLM analysis.

Subsequently, a least-squares mean analysis was performed to assess the DNA tail moment for study subjects expressing one or two susceptible *CYP3A5* and *GSTP1* genotypes, and applying pesticide adjusted for age (Figure 1). In the low and high pesticide exposure groups, individuals possessing no susceptible genotypes had the lowest mean DNA tail moment compared to all other test groups, namely 1.85 ($n = 15$) and 2.17 μm/cell ($n = 18$), respectively. Subjects harboring one susceptible genotype had the slightly higher DNA tail moments (1.91

μm/cell; n = 28 and 2.41 μm/cell; n = 29), respectively, while subjects with two susceptible genotypes in the high pesticide-exposure group had the highest mean tail moment (2.70 μm/cell; n = 1). Amongst the control group, subjects presenting with no susceptible genotypes revealed a mean DNA moment of 1.28 μm/cell (n = 45), and subjects having one susceptible genotype or those having two susceptible genotypes had mean DNA moments of 1.38 μm/cell (n = 56) and 1.55 μm/cell (n = 5), respectively. In addition, the increasing trend in DNA tail moment with the numbers of susceptible genotypes was shown to be statistically obvious at P = 0.07 for the high pesticide-exposure group, and at P = 0.02 for the control group (GLM).

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 (12-14) as well as negative findings (15, 16). The results of the Comet assay presented in this study, together with several previous studies (37, 38), has revealed an increased in DNA damage in the peripheral blood of individuals exposed to complex mixtures of pesticides. The genetic damage demonstrated 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 crosslinks (39). 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 (18). However, the individuals' genetic variability in the enzymes which 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* $G_{-44}G$ or *GSTP1* Ile-Ile genotype had a significantly greater DNA tail moment than those with other genotypes. However it appears 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. Further analysis of *CYP3A5* and *GSTP1* gene combinations, revealed an increased trend for these genotypes to influence DNA tail moment. Importantly, previous studies revealed that pesticide-like organophosphates are primarily metabolized by hepatic cytochrome P450 enzymes to become active intermediate organophosphorus-oxon (19, 20), which may then be hydrolyzed by PON to diethyl phosphate and 4-nitrophenol (20, 21), or conjugated to GSH via catalysis by GSTs (22, 23). CYP3A5 represents at least 50% of the total hepatic cytochrome P450 and metabolizes a wide range of xenobiotics (40). Recently, a $A_{-44}G$ polymorphism in the promoter of the pesudogene *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 (41). Only the subjects with G_{-44} in *CYP3AP1* had normal CYP3A5 expression. Pesticide exposed subjects with *CYP3A5* $G_{-44}G$ genotype had a higher DNA damage in the Comet assay, probably because they had a higher CYP3A5 metabolic activity than those with *CYP3A5* $A_{-44}G/A_{-44}A$ genotypes and therefore an elevated active intermediate levels.

Glutathione S-transferases metabolize various pesticides, many of which are lipophilic electrophiles (22). 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 (BPDE)-DNA adducts in *GSTP1* Ile-Ile carriers when compared to *GSTP1* Ile-Val and Val-Val carriers (42). 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 (43, 44). Thus, GSTP1 may have a dual functionality. Adler et al. (45) 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 Jun kinase (JNK) preventing the phosphorylation of c-jun and subsequent apoptosis. Under conditions of stress, the GSTP1 monomer dissociates from JNK 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 JNK, 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.

Interestingly, those pesticide-exposed individuals who demonstrated with more susceptible genotypes of *CYP3A5* and *GSTP1* were more likely to demonstrate an increased level of DNA damage in our Comet assay. CYP3A5 are involved in the activation of pesticides (19, 20), and GSTP1 acts as detoxifying enzymes for the reactive metabolites of pesticides (22, 23). This indicates that each susceptible genotype may generate a moderate risk for DNA damage; however, when they are combined together, a more prominent risk may develop. It seems that subjects who carry susceptible genotypes of metabolic traits are more likely to express DNA damage mutation when they are exposed to pesticides regardless of high or low pesticide cumulative dose.

In fact, we also tried to minimize some possible biases from ethnicity and life-style by selecting control subjects originating from the same geographic area, and ethnicity of case patients. However, the current active farm population consisted largely of older people in our study area. Most of younger 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 subjects. Previous reports also showed that age is associated with DNA damage (46, 47). 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 reflect 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 (47), 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 to corresponding figures for participants of other studies (37). 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 (17, 18). 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 (ESCODD) (48) 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 ESCODD 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) (49). A dispersion coefficient (standard deviation divided by the mean) of 0.24 for our control subjects was likewise consistent with the analogous figures as revealed by previous studies (50). These findings, to some extent, validate the technique of our Comet assay.

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 handing 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 was obtained without the knowledge of health outcome. Consequently, exposure misclassification is assumed to be non-differential and, if apparent, directed toward an underestimation of the risk for DNA damage.

In summary, the results revealed that metabolic *CYP3A5* and *GSTP1* genes 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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Table 1. Demographic characteristics of pesticide-exposed fruit growers and controls stratified by different intensity of exposure

Variables	Controls	Pesticide exposure	
		Low	High
Number of subjects	106*	43	48
Age (years)	48.9 ± 1.1*	56.7 ± 1.6	55.8 ± 1.7†
Range (years)	21-83	38-79	28-78
Gender: male (%)	38 (35.8%)	18 (41.9%)	27 (56.3%)
Duration of pesticide exposure (years)	0	28.5 ± 2.7	29.8 ± 2.4
Size of orchard (hectares)	0	0.7 ± 0.1	1.7 ± 0.1†
Smoking habit			
Current smoker (%)	15 (14.2%)	7 (16.3%)	11 (22.9%)
Pack-years	2.1 ± 0.6	5.2 ± 2.2	9.0 ± 2.9†

*Data represent numbers of individuals, or means ± SE for continuous variables.

†$P < 0.01$.

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

Genotype		Controls	Pesticide exposure	
Gene	Alleles		Low	High
Number of subjects		106	43	48
CYP3A5	$A_{-44}A$	55 (51.9%)*	26 (60.5%)	26 (54.2%)
	$A_{-44}G$	41 (38.7%)	16 (37.2%)	21 (43.7%)
	$G_{-44}G$	10 (9.4%)	1 (2.3%)	1 (2.1%)
PON1	Gln-Gln	43 (40.6%)	12 (27.9%)	21 (43.7%)
	Arg-Gln	34 (32.1%)	13 (30.2%)	19 (39.6%)
	Arg-Arg	29 (27.3%)	18 (41.9%)	8 (16.7%)
PON2	Cys-Cys	2 (1.9%)	2 (4.6%)	1 (2.1%)
	Cys-Ser	32 (30.2%)	11 (25.6%)	15 (31.2%)
	Ser-Ser	72 (67.9%)	30 (69.8%)	32 (66.7%)
GSTM1	Null	67 (63.2%)	23 (53.5%)	22 (45.8%)
	Non-null	39 (36.8%)	20 (46.5%)	26 (54.2%)
GSTT1	Null	46 (43.4%)	20 (46.5%)	24 (50.0%)
	Non-null	60 (56.6%)	23 (53.5%)	24 (50.0%)
GSTP1	Ile-Ile	56 (52.8%)	27 (62.8%)	30 (62.5%)
	Ile-Val	43 (40.6%)	14 (32.6%)	16 (33.3%)
	Val-Val	7 (6.6%)	2 (4.6%)	2 (4.2%)

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

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

Variables	Controls		Pesticide exposure			
			Low		High	
	n	Mean ± SE	*n*	Mean ± SE	*n*	Mean ± SE
All	106	1.33 ± 0.03	43	1.92 ± 0.04	48	2.35 ± 0.06*
Age						
≥ 52 years	35	1.39 ± 0.06	28	1.94 ± 0.05	27	2.53 ± 0.08*
< 52 years	71	1.30 ± 0.03	15	1.88 ± 0.06	21	2.11 ± 0.08
Gender						
Males	38	1.33 ± 0.04	18	1.89 ± 0.06	27	2.36 ± 0.08
Females	68	1.32 ± 0.04	25	1.94 ± 0.05	21	2.33 ± 0.10
Smoking status						
> 10 pack-years	7	1.36 ± 0.11	6	1.88 ± 0.10	11	2.20 ± 0.10
≤ 10 pack-years	99	1.32 ± 0.03	37	1.92 ± 0.04	37	2.39 ± 0.08
CYP3A5						
$G_{-44}G$	10	1.44 ± 0.12	1	2.34	1	2.79
$A_{-44}G$	41	1.33 ± 0.05	16	1.89 ± 0.06	21	2.31 ± 0.10
$A_{-44}A$	55	1.30 ± 0.02	26	1.91 ± 0.05	26	2.36 ± 0.09
PON1						
Gln-Gln	43	1.28 ± 0.02	12	1.93 ± 0.06	21	2.38 ± 0.09
Arg-Gln	34	1.35 ± 0.05	13	1.92 ± 0.07	19	2.43 ± 0.10†
Arg-Arg	29	1.37 ± 0.12	18	1.90 ± 0.07	8	2.07 ± 0.18
PON2						
Cys-Cys	2	1.38 ± 0.17	2	2.02 ± 0.37	1	2.87
Cys-Ser	32	1.27 ± 0.03	11	1.99 ± 0.08	15	2.30 ± 0.11
Ser-Ser	72	1.35 ± 0.04	30	1.88 ± 0.04	32	2.35 ± 0.08
GSTM1						
Null	67	1.35 ± 0.04	23	1.91 ± 0.05	22	2.34 ± 0.09
Non-null	39	1.29 ± 0.03	20	1.93 ± 0.06	26	2.35 ± 0.09
GSTT1						
Null	46	1.34 ± 0.05	20	1.96 ± 0.05	24	2.27 ± 0.10
Non-null	60	1.32 ± 0.03	23	1.88 ± 0.05	24	2.42 ± 0.08
GSTP1						
Ile-Ile	56	1.37 ± 0.05	27	1.93 ± 0.05	30	2.45 ± 0.07‡
Ile-Val	43	1.27 ± 0.02	14	1.87 ± 0.06	16	2.22 ± 0.13
Val-Val	7	1.30 ± 0.05	2	2.09 ± 0.30	2	1.77 ± 0.02

NOTE: Values are in μm/cell. Comparison amongst different pesticide exposure groups conducted with ANOVA, and comparison between different age, smoking status, and genotype groups conducted with *t*-test, respectively.

$^{*}P < 0.01$. $^{\dagger}P = 0.08$. $^{\ddagger}P = 0.03$.

Table 4. Multiple regression model for tail moment per cell

Variables	Regression coefficient	SE	*P*-value
Intercept	1.15	0.07	< 0.01
Age: ≥ 52 vs. < 52 (years)	0.17	0.05	< 0.01
Gender: Male vs. Female	-0.01	0.05	0.85
Smoking status: > 10 vs. ≤ 10 (pack-years)	-0.04	0.08	0.62
Pesticide exposure			
High vs. Control	1.00	0.06	< 0.01
Low vs. Control	0.54	0.06	< 0.01
Genotyping			
CYP3A5: $G_{-44}G$ vs. $A_{-44}G/A_{-44}A$	0.21	0.09	0.03
PON1: Arg-Gln/Gln-Gln vs. Arg-Arg	0.01	0.05	0.79
PON2: Cys-Cys vs. Cys-Ser/Ser-Ser	0.20	0.14	0.17
GSTM1: Null vs. non-null	0.03	0.05	0.54
GSTT1: Non-null vs. Null	0.02	0.05	0.47
GSTP1: Ile-Ile vs. Ile-Val/Val-Val	0.12	0.05	0.01

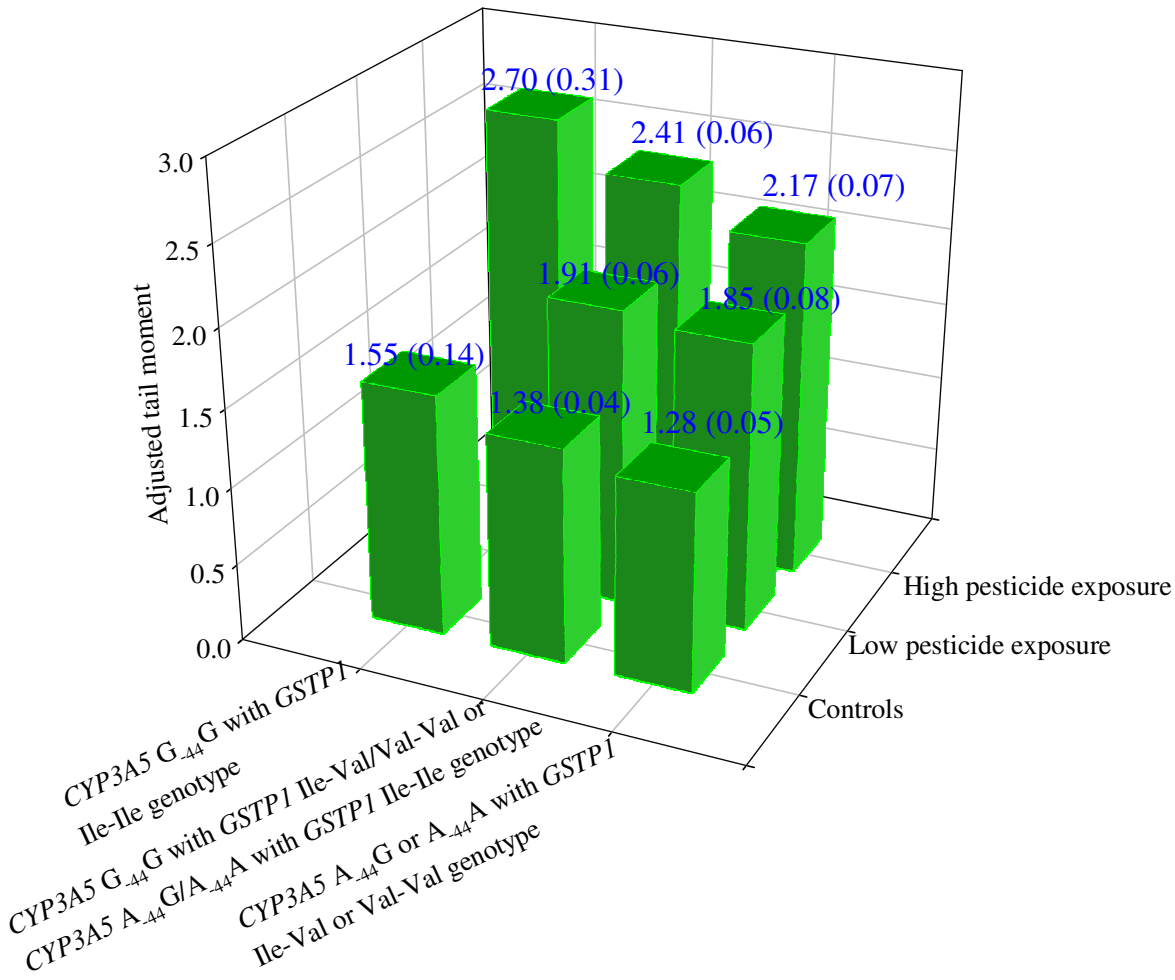

Figure 1. Adjusted mean tail moment according to *CYP3A5* and *GSTP1* genotypes and to pesticide exposure adjusted for age. Standard error is shown in parentheses.